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The overall goals of our Department of Defense proposal were 1.) to demonstrate that the binding of the growth factor, heregulin, to ErbB3 stimulated Neu/ErbB2 tyrosine kinase activity through the formation of a Neu/ErbB2-ErbB3 heterodimer and 2.) to identify signaling activities that were activated by Neu/ErbB2 and/or related tyrosine kinases. We have been able to demonstrate that heregulin binds to ErbB3 and stimulates the formation of Neu/ErbB2-ErbB3 heterodimers in intact cells. We have also shown that Neu/ErbB2 forms heterodimers with other members of the EGF receptor family in cells, resulting in the activation of Neu/ErbB2 tyrosine kinase activity. Moreover, we have found that heregulin stimulates the formation of Neu/ErbB2-EGF receptor (secondary) dimers, as well as Neu/ErbB2-ErbB3 (primary) dimers, thus increasing the diversity of signaling activities that can be initiated by heregulin. We have also identified and characterized two signaling activities that are specifically activated by different growth factors, namely the EGF-stimulated tyrosine phosphorylation of the c-Cbl proto-oncogene product in human breast cancer cells as well as in a canine model system that we have been evaluating, and the heregulin-stimulated activation of the RNA-cap-binding protein, CBP20. The latter finding highlights a novel nuclear end-point for heregulin-coupled signal transduction.

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FOREWORD

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Robert Crine 8/11/98

PI - Signature Date

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INTRODUCTION

The studies outlined in the original proposal were aimed at determining the molecular basis underlying the actions of the Neu/ErbB2 tyrosine kinase and its involvement in the development and progression of breast cancer. Within this section of the final report (for the period July 15, 1994-July 15, 1998), I will discuss the background of the research problem (within the context of previous work performed by our laboratory and other laboratories), the purpose of the proposed research and the methods of approach that have been used.

Background of the Research Problem- It is well accepted that cancer is the outcome of a loss of regulation at one or more points in a growth factor-coupled signal transduction pathway. In many cases of breast cancer, the activation of a growth factor receptor first called Neu (1), and then more recently ErbB2 (2) or HER2 (for the human protein), may represent the first step in an aberrant signaling pathway that is responsible for this disease. The Neu/ErbB2 tyrosine kinase is a member of a large superfamily of transmembrane receptors. Members of this superfamily that share the highest degree of sequence similarity with Neu/ErbB2 make up the subclass 1 receptor tyrosine kinases and include the epidermal growth factor (EGF) receptor (3,4), the ErbB3 (HER3) protein (5,6), and the ErbB4 (HER4) protein (7). Each of these receptors is comprised of a single polypeptide chain (M_r ~170-180 kDa) that includes a cysteine-rich extracellular (growth factor binding) domain, a single transmembranal helix of 23 amino acids, and a cytoplasmic tyrosine kinase domain that contains a number of potential autophosphorylation sites.

The main goal underlying the DOD proposal (DAMD17-94-J-4123) was to understand the molecular regulation of the Neu/ErbB2 tyrosine kinase, with a particular emphasis on the mechanisms responsible for its activation and signaling functions. The oncogenic capability of the *neu* gene was first identified in chemically-induced rat neuroblastomas (1). A single point mutation that resulted in the substitution of a glutamic acid for a valine residue at position 664 within the transmembrane domain of the Neu tyrosine kinase was shown to result in oncogenic activation. Thus far, no mutation analogous to that which was responsible for the oncogenicity of the rat protein has been detected in the human counterpart of Neu (i.e. the ErbB2 or HER2 proteins). However, given the correlation between the levels of ErbB2 and the poor prognosis for breast cancer patients, it has been assumed that high expression of ErbB2 results in an over-stimulation of a signaling pathway that causes the proliferation of mammary cells.

Because of the apparent importance of the regulation of the Neu/ErbB2 tyrosine kinase activity, a great deal of effort was directed toward the isolation and identification of a ligand/growth factor for this receptor. It originally was reported that a family of glycoproteins called the heregulins (also referred to as the Neu-differentiation-factor or NDF) served as ligands for Neu/ErbB2 in breast cancer cells (8-11). Both NDF and the heregulins were shown to stimulate tyrosine phosphorylation in breast cancer cells. However, not all cells that expressed Neu/ErbB2 bound heregulin or NDF (e.g. fibroblasts). This indicated that breast cancer cells must possess a component that enables Neu/ErbB2 to respond to heregulin. We found that the same was true for rat pheochromocytoma (PC12) cells which undergo neurite extension in response to the activation of the Neu/ErbB2 receptor (see below). Given the tendency of members of the EGF receptor family to form heterodimers, it was tempting to speculate that another member of the family (i.e. aside from Neu/ErbB2) initially bound heregulin (or NDF) and then formed a heterodimer with Neu/ErbB2 and conferred responsiveness to heregulin.

Using insect cell-expressed tyrosine kinases, we found that ErbB3, which is ~60% identical to the EGF receptor and Neu/ErbB2, served as a receptor for heregulin (12). This led us to propose the following hypothesis. Heregulin binding to ErbB3 promotes the formation of an ErbB3-Neu/ErbB2 heterodimer which results in the trans-phosphorylation of ErbB3 by Neu/ErbB2. The phosphorylation of ErbB3 could then initiate signaling pathways that impact on cell growth. In this regard, we felt that ErbB3 might represent a new class of receptor molecules that provides the potential for dual regulation in growth factor signaling, specifically, by enabling other receptors (i.e. Neu/ErbB2) to respond to unique ligands (heregulins) and by serving as a target for trans-phosphorylation reactions that elicit the recruitment of specific substrates. Work by

Plowman and colleagues similarly showed that ErbB4 also was capable of serving as a receptor for heregulin (13) and so presumably heregulin-simulated ErbB4-Neu/ErbB2 interactions could also occur and give rise to specific signaling activities.

Outline and Purpose of the DOD Research- The overall goal of the studies outlined in the original proposal was to understand the molecular basis underlying the actions of Neu/ErbB2 and heregulin in different cells. After the submission of the DOD proposal, we made the interesting observation that while ErbB4 is capable of autophosphorylation and tyrosine kinase activity in a heregulin-stimulated manner, ErbB3 is not (see below). We then showed that ErbB3, itself, was capable of little or no tyrosine kinase activity (14), either in the presence or absence of added heregulin. This most likely reflects the fact that ErbB3 differs from all other kinases at four positions (in particular there is an asparagine residue at position 834 which is an aspartic acid in all other kinases and is thought to be an essential residue in the active center of the cyclic AMP-dependent protein kinase). We found that the co-expression of ErbB3 and Neu/ErbB2 in COS cells reconstituted a higher affinity heregulin receptor (K_d ~50 pM compared to a K_d of ~1 nM for ErbB3 alone) and a heregulin-stimulated phosphorylation of both the Neu/erbB2 and ErbB3 proteins (see below; also ref. 15). Taken together, these results supported our original hypothesis that the binding of heregulin to the ErbB3 receptor protein stimulates the formation of a heterodimer between ErbB3 and Neu/ErbB2 which enables heregulin to bind with high affinity and to stimulate the tyrosine kinase activity of Neu/ErbB2. Because of the presence of a number of candidate (tyrosine) phosphorylation sites within the carboxyl terminal domain of ErbB3, and because these tyrosine residues when phosphorylated would be predicted to bind tightly to the SH2 domains of other candidate signaling molecules, it seemed likely that ErbB3 would serve as an adapter molecule in the Neu/ErbB2 signaling pathway. Presumably, the heregulin-stimulated phosphorylation of ErbB3, within a ternary complex consisting of heregulin-ErbB3-Neu/ErbB2, would serve to recruit the next protein(s) in the signaling cascade. We originally proposed that one such signaling protein was the Src kinase because it had been reported to play a role in the development of some breast cancers (16) and because phosphorylated ErbB3 was predicted to form a tight complex with the SH2 domain of Src (17). We were unable to find evidence for a stable ErbB3-Src complex in various cell lines that we examined; however, we did find that another SH2 domain-containing protein, the 85 kDa regulatory subunit of the PI 3-kinase, formed a specific complex with ErbB3 in a heregulin-dependent manner in PC12 cells (see below). We also showed that activated (transforming) Neu/ErbB2 was able to induce neurite extension in PC12 cells in a manner analogous to the nerve growth factor (NGF) receptor (trk) (18) and that heregulin stimulated this response in PC12 cells expressing normal Neu/ErbB2.

Thus, we suspected that in PC12 cells, ErbB3 or a related protein component, acting in conjunction with Neu/ErbB2, was probably responsible for conferring responsiveness to heregulin. This led us to verify the occurrence of these signaling cascades in neuronal cells, where Neu/ErbB2 may play a key role in development, as well as in mammary cells where Neu/ErbB2 may be involved in the regulation of cell growth and in the development of breast cancer. To do this, we have used a combination of protein biochemistry and recombinant DNA technology to dissect the Neu/ErbB2 signaling pathway. Through cDNA transfection approaches, we have introduced the cDNAs for wild type and mutant Neu/ErbB2 and ErbB3 into cells to determine the potential for interaction between these proteins in a heregulin-dependent manner. These studies also depended heavily on specific antibody reagents against the Neu/ErbB2 and ErbB3 proteins that became available to us. These antibodies were used in immunoprecipitation and Western blotting experiments to verify the existence of different signaling complexes containing the Neu/ErbB2 and ErbB3 proteins. We also used *Spodoptera frugiperda* expression systems to generate recombinant proteins (e.g. insect cell-expressed Neu/ErbB2 and ErbB3) for biochemical characterization. As these studies progressed we made a number of unexpected but nonetheless exciting discoveries regarding receptor tyrosine kinase signaling. One involved the identification of the c-Cbl protein as a major phospho-substrate for the EGF receptor in human breast cancer cells. This in turn led us to the finding that a novel family of proteins, called Cool (for Cloned-out-of-a-library), which serve as key regulators of signaling via the small G proteins Cdc42 and Rac

and interact with the c-Cbl-related breast cancer protein Cbl-b, may serve to interface Cdc42 and/or Rac to receptor tyrosine kinases in human breast cancer cells. More recently, while searching for novel nuclear activities that are stimulated by heregulin, we discovered an 18 kDa heregulin-stimulated GTP-binding activity which appears to be present in the nucleus of all cells. We have shown that this protein is identical to an RNA cap-binding protein, CBP20, which binds the ^{7}methyl guanosine cap structure on RNAs transcribed by RNA polymerase II and appears to be essential for an early step in the splicing of precursor messenger RNA. Thus, we have uncovered a new end-point for heregulin and/or Neu/ErbB2 signaling to the nucleus, namely the regulation of RNA processing. We expect that each of these findings will provide new information regarding how cell growth is regulated in mammary cells and will enable us to identify additional EGF-, heregulin- and/or Neu/ErbB2-responsive signaling activities. We plan to continue to pursue each of these leads after the termination of the DOD award, because we are quite excited about the possibilities. In addition, we have been examining the potential for an animal model system, based on the development of spontaneous mammary carcinomas in the dog. If we demonstrate that the interactions between Cool and Cbl-b are altered or p18 is upregulated in canine tumor cells, the longer term goal will be to identify reagents that interfere with these activities and ultimately test their effects in trials on dogs that have been diagnosed with this disease. The hope is that the information that we gain from these studies will lead to new methods for screening or to the development of novel strategies (e.g. targeted at RNA processing) for therapeutic intervention against breast cancer.

BODY

Working Hypothesis/Description of Objectives- The original proposal submitted to the Department of Defense Breast Cancer Initiative was largely based on our findings that the Neu/ErbB2 protein is not a receptor for heregulin, as originally thought. Rather, the related ErbB3 protein serves as a receptor for heregulin and thus may play an essential role in conferring responsiveness of Neu/ErbB2 to heregulin (through the formation of ErbB3-Neu/ErbB2 heterodimers). Given this hypothesis, we proposed to verify that the cellular actions of heregulin were in fact mediated by its initial binding to the ErbB3 protein (Aim 1). We also wanted to show that heregulin-stimulated ErbB3-Neu/ErbB2 interactions occurred in intact cells (Aim 2). Once these points were verified, we hoped to determine what happens after the heregulin-stimulated ErbB3-Neu/ErbB2 interaction, i.e. identify other cellular proteins that act in the signaling pathway (Aim 3). To achieve these aims, we initially felt that it would be necessary to generate suitable expression systems for ErbB3 and Neu/ErbB2 in insect cells for *in vitro* studies and cell lines and antibody reagents for studying these signaling pathways in intact cells. These represented the first four tasks listed under "Statement of Work" in the original proposal. While these various aims were being pursued, we also intended to determine whether the EGF receptor might play an important role in signaling pathways relevant to the development of mammary carcinomas, for example, through the EGF-stimulated activation of Neu/ErbB2. Along these lines, it seemed attractive to contemplate the possibility that both EGF and heregulin (through distinct mechanisms) could positively impact on Neu/ErbB2 activity. Finally, the long range goal of the proposal was to use the information generated from these molecular studies to begin to design possible therapeutic strategies. Specifically, we hoped to determine that spontaneous mammary carcinomas that occur in the dog would serve as a suitable model for human breast cancer (Aim 4 and the final task under "Statement of Work").

During the past four years, we have been able to achieve many of our initial objectives. We have shown that heregulin-stimulated Neu-ErbB3 heterodimer formation occurred in cells and that this led to the activation of Neu, the (tyrosine) phosphorylation of ErbB3, and the formation of a complex between phosphorylated ErbB3 and p85. We also showed that other receptor dimer combinations were capable of forming (these included EGFR-Neu/ErbB2 and EGFR-ErbB3 complexes). Each of these heterodimers were capable of initiating distinct signaling pathways, such that only the heregulin-stimulated ErbB3-Neu/ErbB2 heterodimer resulted in the formation of a specific complex between ErbB3 and p85. The complexity and diversity of signaling activities

originating from growth factor-stimulated receptor dimerization events was reinforced by our findings that secondary receptor dimers can arise from primary receptor dimerization events (also, see below). As alluded to in the preceding section, we also identified two potentially new signaling pathways, one that is specifically initiated by EGF and involves the Cbl proteins and a second that is specifically initiated by heregulin and culminates in the activation of the 18 kDa RNA cap-binding protein, CBP20. The latter represents to our knowledge the only known nuclear end-point of receptor tyrosine kinase signaling that is specific for heregulin (versus EGF). We have also been able to show that the same signaling activities attributed to the EGF receptor and Neu/ErbB2 in human breast cancer cells can be found to be operating in dog mammary carcinomas, suggesting that the dog may ultimately provide a valuable animal model for the human disease. In obtaining these various lines of new information, we have when necessary deviated from the original plan, either because reagents unexpectedly became available or because a particular line of study seemed more relevant (e.g. searching for novel nuclear activities regulated by heregulin-signaling). For example, antibodies became commercially available (as well as from collaborators) for Neu/ErbB2 and ErbB3 rather quickly, which allowed us to circumvent the task of preparing these reagents and move more quickly into cellular studies. We also found that studies in cells yielded more meaningful information than *in vitro* studies, using reconstituted phospholipid vesicle systems, and so we placed more emphasis on the cellular work. All of this will be described in more detail in the following sub-sections, which will be presented in the order that most closely corresponds to the original aims of the proposal. All publications resulting from this work are indicated in bold and are enclosed in the appendix.

Progress of Research: Experimental Procedures

This section will contain some of the more routinely used experimental procedures that are relevant to the results described below. For more details or additional methods, see the attached manuscripts.

1. Cloning and expression of members of the EGF receptor family in insect cells- An ~500-bp fragment of the *erbB3* cDNA was inadvertently cloned from a bovine brain cDNA library as a side product of an independent polymerase chain reaction. This fragment was then used to isolate a cDNA corresponding to the entire coding region of bovine ErbB3, which exhibited 94% amino acid identity with the human protein (5,6) over the entire open reading frame. The cDNAs encoding the human EGF receptor, rat Neu/ErbB2, and bovine ErbB3 were inserted into the pAcYMP1 insect-cell transfer vector (19) by standard recombinant DNA methods. Recombinant viruses encoding the EGF receptor, Neu/ErbB2, and ErbB3 proteins were prepared by using the BaculoGold kit (PharMingen) according to the directions of the manufacturer and were plaque-purified prior to expression of the recombinant receptors. Sf9 cells were infected with various viruses and harvested after 48-72 hr. Purification of plasma membranes and phosphorylation of the polymer substrate $(E_4Y_1)_n$ [which is a random 4:1 copolymer of glutamic acid and tyrosine, or poly($Glu^{80}Tyr^{20}$); Sigma] by membranes were carried out as previously reported (19). Membrane preparations from cells infected with the different viruses were normalized to protein concentration.

2. Immunoprecipitation and tyrosine kinase assays of insect cell proteins- Phosphorylations assays on insect cell membranes containing recombinant receptors were typically performed in a 100-µl volume in the presence of 5 mM $MnCl_2$ and 20 µM [γ-^{32}P]ATP (~3,000 Cerenkov cpm/pmol) for 10 min at room temperature. The reactions were stopped by the addition of 50 µl of 5X sample buffer (20) and proteins were subjected to SDS/7% PAGE. The model substrates that were most typically used, $(E_4Y_1)_n$ and GST-p85 (glutathione S-transferase fusion protein with the 85-kDa subunit of phosphatidylinositol 3-kinase; purified with glutathione agarose as described in ref. 20a) were present in the phosphorylation assays at concentrations of 0.5 mg/ml and 0.05 mg/ml, respectively. Phosphorylated bands were excised from the dried gels, and the associated radioactivity was then determined by Cerenkov counting. When quantitating the

phosphorylation of the synthetic peptide $(E_4Y_1)_n$, a segment of the gel lane corresponding to the 43-kDa to 105-kDa region was excised.

3. Recombinant heregulins- All experiments described below used a 68 amino acid fragment of heregulin-β1 (residues 177 through 244) which corresponds to the EGF-like domain. This fragment (which for simplicity will be referred to as heregulin) was expressed in *Escherichia coli* and purified to homogeneity as described previously (8). We have found that this fragment matches the abilities of the full-length versions of heregulin-β1 and heregulin-α, that are produced and purified from mammalian cells, to bind ErbB3 and exhibits the high affinity binding induced by the presence of Neu/ErbB2 together with ErbB3. Thus, in agreement with previous findings (8), the EGF-like domain is sufficient for heregulin binding; in addition, the α and β1 isoforms of heregulin do not show any detectable differences in binding capability or specificity.

4. Expression vectors and transfections of ErbB3 and Neu/ErbB2 in COS-7 cells- The cDNA encoding bovine ErbB3 was subcloned into the pRK7 expression vector containing the cytomegalovirus promoter (10). The cDNA encoding the human Neu/ErbB2 tyrosine kinase, also in pRK7, was generated by Sharon Erickson, Genentech, Inc. COS-7 cells (ATCC CRL 1651) were transfected using the LipofectAMINE™ protocol obtained from Life Technologies, Inc. Cells were transfected in 15-cm plates using a total of 18 μg of DNA. When cells were transfected with both Neu/ErbB2 and ErbB3, 9 μg of each expression plasmid was used. Single transfections were performed (for 24-36 h) using 9 μg of the specific cDNA-containing plasmid and 9 μg of the pRK7 vector control plasmid.

5. Cells, growth factors and antibodies for PC12 cell studies- Rat pheochromocytoma (PC12) cells (obtained from Dr. M. Chao, Cornell University Medical School) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. Anti-phosphotyrosine antibody (PY20) was obtained from Transduction Laboratories, the anti-Neu/ErbB2 antibody, Ab-3, was from Oncogene Science Inc. (Manhasset, NY), and anti-ErbB4 was from Santa Cruz Biotechnology. The anti-EGF receptor antibody, 13A9, was obtained from Genentech (San Francisco, CA). Anti-p85 antibody was a generous gift from Drs. L. Cantley and C. Carpenter (Harvard Medical School). The generation and use of the anti-ErbB3 monoclonal antibody that was used in the PC12 cell studies, 2F12 (Neo Markers, Fremont, CA), have been described elsewhere (22).

6. Transfections into PC12 cells- For PC12 cell transfections, the cDNA encoding bovine ErbB3 (12) was subcloned into pcDNAI/neo (Invitrogen) and then introduced into the cells by the LipofectAMINE™ method. Stably transfected cells were selected using 400 μg/ml (active) of the antibiotic G418 (Geneticin, Life Technologies, Inc.).

7. Growth factor treatment, immunoprecipitations and Western blot analyses- Cells were grown to near confluence in Dulbecco's modified Eagle's medium plus 10% horse serum and 5% fetal bovine serum on 150-mm tissue culture dishes. 16-20 h prior to growth factor treatment, the media were changed to Dulbecco's modified Eagle's medium with 0.1% fetal bovine serum. The cells were then removed from the dishes by triteration in Hanks' balanced salt solution without calcium or magnesium, pelleted (500 rpm for 5 min), and resuspended in Dulbecco's modified Eagle's medium without serum. Cells were dispensed at 1.0 x 10^7/1.5 ml/microfuge tube. Heregulin (20 nM) or EGF (100 ng/ml) was added, and the cells were incubated at 37°C for 5 min. The treatment was terminated by plunging microfuge tubes containing the cells into ice, followed by immediate centrifugation to pellet the cells and aspiration of the factor-containing supernates. The cell pellets were then lysed in Tris-buffered saline with 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EGTA, 40 mM sodium fluoride, 100 μM ammonium molybdate, and 1 mM sodium orthovanadate. The lysates were incubated for 15 min on ice, after which insoluble materials were pelleted in a microfuge (10 min). Each supernate was then incubated for 2 h on ice with 30 μl of a 50%

suspension of protein A-Sepharose (Sigma) and the indicated antibodies. The immune complexes were pelleted, boiled in 40 µl of Laemmli sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis (8% acrylamide), and transfered to nitrocellulose. The blots were blocked in 3% bovine serum albumin in Tris-buffered saline plus 0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4°C in primary antibody as indicated. Blots were analyzed by ECL (Amersham).

Progress of Research: Results and Discussion

We felt that an important first step in our proposed studies was to obtain data supporting the idea that heregulin binding to ErbB3 stimulated ErbB3-Neu/ErbB2 heterodimer formation. Following the submission of our proposal to the DOD, we were able to obtain such data (actually faster than we originally anticipated). This came from studies examining the possible interactions between ErbB3 and Neu/ErbB2 after the co-expression of these tyrosine kinases in COS-7 cells (15) and in insect cells (Platko and Cerione, unpublished data). COS-7 cells that were transfected with *erbB3* cDNA, alone, using the lipofectamine method, displayed a single class of heregulin binding sites with an apparent K_d of 1.9 nM. No binding was detected in COS cells that expressed Neu/ErbB2 alone. We also detected little or no heregulin-stimulated tyrosine phosphorylation in COS cells that expressed either ErbB3 or Neu/ErbB2 alone. However, the co-expression of ErbB3 and Neu/ErbB2 (where ErbB3 was expressed in 10 fold excess over Neu/ErbB2) reconstituted a high affinity heregulin binding interaction (K_d ~0.02 nM) and a low affinity interaction (K_d ~ 2 nM)(Figure 1); the latter was similar to what was observed in cells expressing

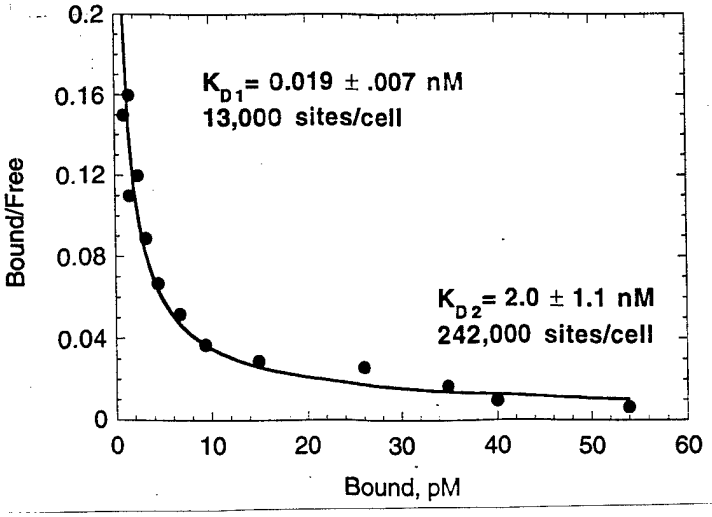


Figure 1: Scatchard analysis of [^{125}I]heregulin binding to COS-7 cells expressing Neu/ErbB2 and ErbB3. A 10-fold excess of *erbB3* expression plasmid relative to *neu/erbB2* was used for COS-7 cell transfections. The recombinant heregulin was iodinated and the binding assays were performed as described in (15). Data taken from Sliwkowski et al. (15).

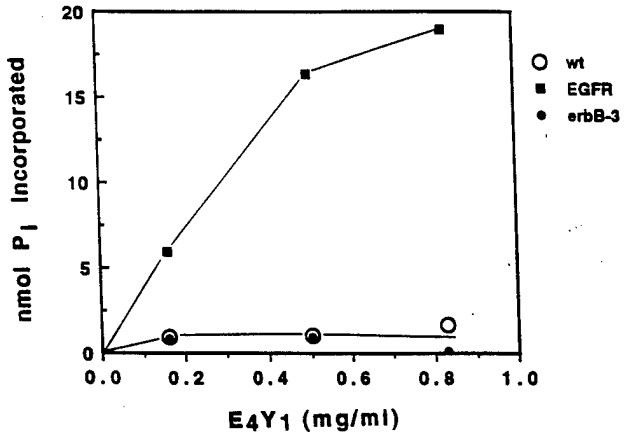


Figure 2: Membranes from Sf9 insect cells infected with wild-type baculovirus (wt) and with recombinant viruses encoding the EGF receptor and ErbB3 were used to phosphorylate the indicated concentrations of the polymer substrate $(E_4Y_1)n$ as described in (14). Data taken from Guy et al. (14).

ErbB3 alone. COS cells that expressed both ErbB3 and Neu/ErbB2 also showed a heregulin-stimulated tyrosine phosphorylation of the ErbB3 and Neu/ErbB2 proteins. We went on to show that the co-expression of ErbB3 and Neu/ErbB2 in insect (Sf9) cells, via baculovirus infection, also resulted in heregulin-stimulated phosphorylations of ErbB3 and Neu/ErbB2, whereas this was not observed in Sf9 cells expressing either receptor alone (Platko and Cerione, unpublished data). Similar data was obtained when ErbB3 and Neu/ErbB2 were solubilized from insect cell membranes and then inserted into phosphatidylcholine lipid vesicles, using procedures that were developed in this laboratory for reconstituting receptor tyrosine kinases (23,24). Thus, these

results provided strong evidence for our working hypothesis that heregulin influences Neu/ErbB2 activity by first binding ErbB3 and then promoting ErbB3-Neu/ErbB2 interactions.

During this time period, we also made the unexpected finding that insect cell-expressed ErbB3 appeared to be kinase-defective (14), such that it lacked detectable autophosphorylation and was at least two orders of magnitude less effective than the EGF receptor in catalyzing the phosphorylation of exogenous substrates such as synthetic tyrosine-containing peptides (Figure 2). Thus, ErbB3 appears to differ from other members of the EGF receptor family and acts more as an adaptor molecule, by binding heregulin and confering heregulin-sensitivity to Neu/ErbB2. The fact that ErbB3 contains 13 tyrosine residues also makes it an attractive substrate for Neu/ErbB2.

Based on these findings, we decided to set out to demonstrate that heregulin-stimulated interactions between ErbB3 and Neu/ErbB2 occur in cells under conditions where heregulin triggers a detectable biological response. We found that rat pheochromocytoma (PC12) cells served as an excellent model system for such studies.

1. Characterization of heregulin-ErbB3 interactions and demonstration of ErbB3-Neu/ErbB2 interactions in cells that yield a heregulin-stimulated response- As outlined above, a major reason for studying the molecular basis underlying the activation of the Neu/ErbB2 tyrosine kinase is the implication that it participates in the development of human cancers (in particular, human breast and cervical cancers). Interestingly, it also has been suspected that Neu/ErbB2 plays a role in neuronal development since a putative ligand/growth factor for Neu/ErbB2, called heregulin or Neu differentiation factor (NDF), is identical to glial growth factor (25) and acetylcholine receptor-inducing activity [ARIA (21)]; it is now known that the heregulins constitute a family of spliced variants and have recently been collectively refered to as the neuregulins. Consistent with a possible involvement of the neuregulins and Neu/ErbB2 in neuronal functions, we have shown that the expression of a transforming version of the Neu/ErbB2 tyrosine kinase (i.e. where the valine residue at position 664 within the transmembranal domain has been changed to a glutamic acid) was capable of stimulating neurite extension in PC12 cells (18). Moreover, we found that the addition of heregulin to PC12 cells that over-expressed the normal Neu/ErbB2 tyrosine kinase also elicited neurite extension. These results thus highlighted two important points. The first was that the activation of Neu/ErbB2, either by a point mutation that increased its tyrosine kinase activity or by the addition of its putative ligand/growth factor, elicited a cellular morphology that was virtually identical to that induced by the nerve growth factor (NGF) receptor (i.e. trk) but distinct from the cellular effects elicited by the EGF receptor. Thus, PC12 cells offer an excellent model system for distinguishing important features of the signaling pathways initiated by activated Neu/ErbB2 versus the activated EGF receptor. A second major implication is that the addition of heregulin leads to the activation of the Neu/ErbB2 tyrosine kinase in PC12 cells. Thus, like breast cancer cells, PC12 cells must contain the necessary components for the stimulatory regulation of Neu/ErbB2 by heregulin.

Given these important features of PC12 cells, we have used these cells as a model system to address two important points. The first was to determine if the ErbB3 protein participated with Neu/ErbB2 in eliciting heregulin-stimulated neurite extension in PC12 cells. The ability to demonstrate that heregulin interactions with ErbB3 represented a critical first step toward eliciting a real biological response, would provide compelling support for the working hypothesis which formed the backbone of our proposal to the DOD. The second point was to identify differences in the signaling cascades initiated by heregulin and EGF. We felt that the PC12 cell system offered a unique opportunity to study differences in EGF- and heregulin-stimulated signaling, because only the latter led to neurite extension.

We first found that heregulin stimulated the tyrosine phosphorylation of the endogenous ErbB3 protein in PC12 cells, as visualized by immunoprecipitating ErbB3 with a specific monoclonal antibody and then Western blotting with anti-phosphotyrosine (compare lanes 3 and 4 in panel B of Figure 3, below, also see **Gamett et al., 1995; Appendix I**). This phosphorylation, as well as a heregulin-stimulated phosphorylation of Neu/ErbB2 (which appeared as a doublet), was greatly enhanced in cells over-expressing Neu/ErbB2 (see lanes 7 and 8 in panels A and B in Figure 3). The ErbB3 phospho-band was broad and appeared to contain a

component with lesser electrophoretic mobility than that found in the anti-Neu/ErbB2 precipitates. This will be considered further below. It also is important to note that while the ErbB4 protein (another putative receptor for heregulin) could be detected by Western blotting anti-phosphotyrosine precipitates with a specific anti-ErbB4 antibody, the tyrosine phosphorylation of ErbB4 was not stimulated by heregulin in PC12 cells. We also found that over-expressing the ErbB3 protein in PC12 cells led to heregulin-stimulated tyrosine phosphorylation of Neu/ErbB2 and ErbB3 (to greater extents than observed in parental PC12 cells) and a heregulin-stimulated neurite extension (**Gamett et al., 1995; Appendix I**), similar to the phenotypes obtained upon heregulin addition to cells over-expressing the normal Neu/ErbB2 tyrosine kinase or following the expression of transforming Neu/ErbB2 in PC12 cells.

Two important questions concerned whether the Neu/ErbB2 and ErbB3 proteins actually formed a complex in PC12 cells and if this complex formation was stimulated by heregulin. The results of immunoprecipitation experiments using an anti-Neu antibody indicated that the addition of heregulin either to PC12 cells that over-express Neu/ErbB2 (see lanes 3 and 4 in panel B of Figure 3), or PC12 cells that over-express ErbB3 (**Gamett et al., 1995; Appendix I**), led to the precipitation of the Neu/ErbB2 and ErbB3 proteins. Thus, overall, these results were consistent with a scheme where heregulin-stimulated heterodimer formation between Neu/ErbB2 and ErbB3 results in increased tyrosine phosphorylation of the Neu/ErbB2 and ErbB3 proteins and accounts for the ability of PC12 cells to respond to this growth factor. However, the fact that substantially more of the heregulin-stimulated ErbB3-Neu/ErbB2 complex was found in cells over-expressing Neu/ErbB2 or ErbB3 compared to the parental PC12 cells suggests that the affinity of Neu/ErbB2 for ErbB3 is relatively weak, such that sufficient complex formation can only be detected by immunoprecipitation under conditions where the amount of one or the other of these proteins is relatively high.

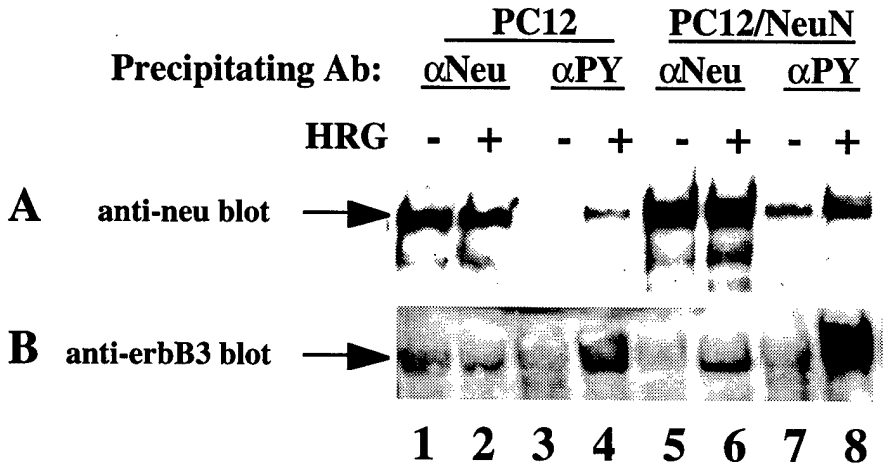


Figure 3: Heregulin-stimulated tyrosine phosphorylation of Neu/ErbB2 and ErbB3. Parental PC12 cells or PC12 cells transfected with wild-type Neu/ErbB2 (PC12/NeuN) were incubated for 5 minutes with (+) or without (-) 10 nM heregulin (HRG) in serum-free medium and then cell lysates were prepared as described in Gamett et al., 1995, Appendix. The lysates were divided equally for immunoprecipitation with antibodies against Neu (αNeu) or phosphotyrosine (αPY). A.) The precipitates were analyzed by immunoblotting with antibody against Neu/ErbB2. B.) This represents a separate experiment in which the precipitates were analyzed by immunoblotting with the 2F12 monoclonal antibody against ErbB3. Data taken from Gamett et al., 1995; Appendix.

Since various lines of evidence (27) have suggested that one of the primary effector/targets for phosphorylated ErbB3 is the 85 kDa regulatory subunit (p85) of the PI 3-kinase, we examined whether heregulin addition to PC12 cells might lead to the formation of a stable ErbB3-p85 complex. In addition, given that we had evidence for the existence of two or more phosphorylated forms of ErbB3 in heregulin-treated PC12 cells, while only a single ErbB3 phospho-band

appeared to associate with Neu/ErbB2, we were interested in determining whether there were distinct roles for the different (phospho)forms of ErbB3. Immunoprecipitation experiments using a specific (precipitating) anti-p85 antibody, followed by Western blotting with a specific anti-ErbB3 antibody, provided evidence for a direct interaction between these proteins. This interaction was most evident in PC12 cells over-expressing Neu/ErbB2 and was heregulin-stimulated. When comparing the results of anti-phosphotyrosine immunoprecipitates with those of anti-p85 immunoprecipitates (where in both cases the resuspended precipitates were blotted with anti-ErbB3 antibody), we found that the ErbB3 band that co-precipitated with p85 was identical in mobility to the slowest mobility ErbB3 band detected in anti-phosphotyrosine precipitates (see the double arrow in Figure 4, below). However, this slow-mobility ErbB3 band was not detected in anti-Neu/ErbB2 immunoprecipitates. These results suggested that the ErbB3 protein was phosphorylated at multiple tyrosine residues in a heregulin- and Neu/ErbB2-dependent manner and that one of these phosphorylated ErbB3 species formed a stable complex with Neu/ErbB2 but not with p85.

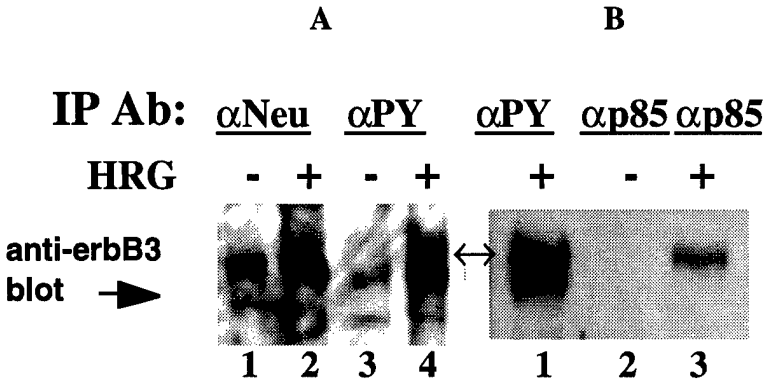
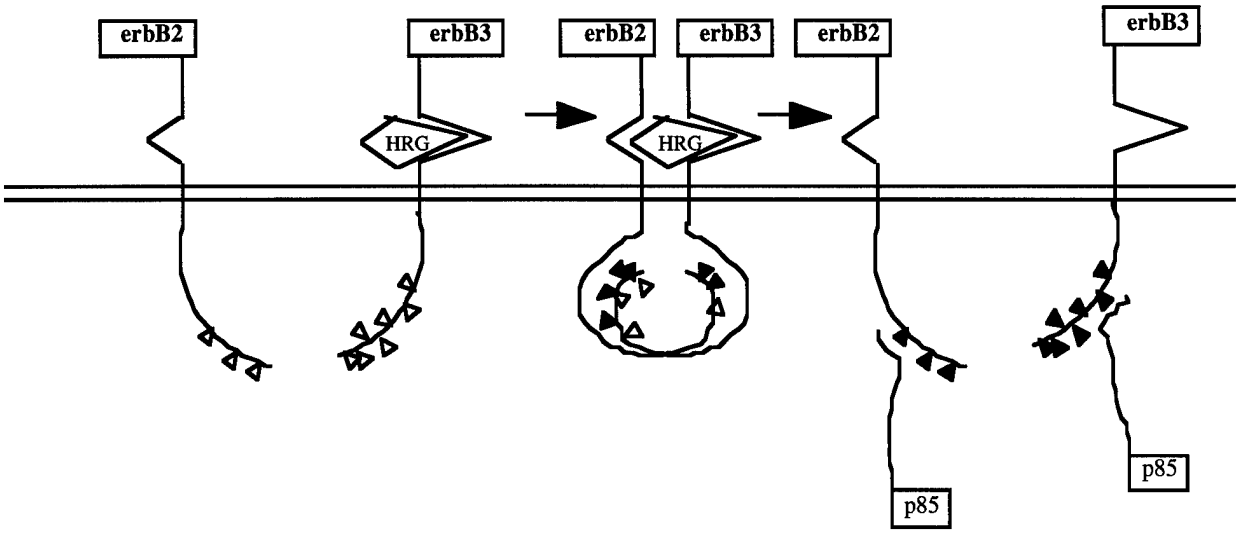


Figure 4: Anti-ErbB3 immunoblots showing association of different forms of ErbB3 protein with Neu/ErbB2 and p85. A.) Lysates from PC12 cells transfected with Neu/ErbB2 were immunoprecipitated with anti-Neu/ErbB2 (αNeu) (lanes 1 and 2) and the supernatants after this precipitation were immunoprecipitated with anti-phosphotyrosine (αPY) (lanes 3 and 4). B.) Lysates of heregulin (HRG)-stimulated cells were divided equally for immunoprecipitation with antibodies against antiphosphotyrosine or p85 (αp85). The double arrow indicates the more slowly migrating form of ErbB3. IP Ab refers to immunoprecipitation antibody. Data taken from Gamett et al., 1995; Appendix.

These results appear to be consistent with the simple scheme shown below. The binding of heregulin (HRG) to the ErbB3 protein stimulates the formation of an ErbB3-Neu/ErbB2 heterodimer. This results in the increased tyrosine phosphorylation of Neu/ErbB2, at multiple sites (shown by the triangles), and the cross-phosphorylation of multiple tyrosine residues on ErbB3. At the present time, it is not clear how heregulin stimulates the tyrosine phosphorylation of the Neu/ErbB2 protein. While one obvious possibility would have been the trans-phosphorylation of Neu/ErbB2 by ErbB3, this seems unlikely because ErbB3 shows little or no tyrosine kinase activity (as discussed on pages 10-11; also see ref. 14). However, it cannot be ruled out that within an ErbB3-Neu/ErbB2 heterodimer, ErbB3 is still able to trans-phosphorylate Neu/ErbB2, despite its weak kinase activity, because of the immediate proximity of the substrate (Neu/ErbB2). An alternative possibility is that another tyrosine kinase (e.g. Src) joins the ErbB3-Neu/ErbB2 complex and phosphorylates Neu/ErbB2 (however, see below). One of the initial aims of the DOD proposal was to determine whether c-Src formed a complex with ErbB3, based on considerations of primary sequence which suggested that tyrosine phosphorylated ErbB3 may serve as a binding partner for the SH2 domain of c-Src. However, the ability to detect an ErbB3-Src interaction in any of a number of human breast cancer cells (including SKBR3, MD-MBA-453 cells and MD-MBA-468 cells) or in PC12 cells have yielded negative results. A third possibility is that protein-protein interactions between Neu/ErbB2 and ErbB3 result in an activation of Neu/ErbB2 tyrosine



kinase activity. Whatever the mechanism, once activated, Neu/ErbB2 should be able to effectively trans-phosphorylate ErbB3. The timing of this trans-phosphorylation event is critical, since the extent of tyrosine phosphorylation of ErbB3 determines whether ErbB3 remains complexed with Neu/ErbB2 or forms a new complex with p85. These findings then raise the interesting possibility that the heregulin-stimulated trans-phosphorylation of ErbB3, by Neu/ErbB2, leads to the dissociation of ErbB3 from Neu/ErbB2 and results in the specific binding of ErbB3 to a potential target, p85. In this regard, the mechanism would be similar to those commonly proposed for hormone receptor/G protein-mediated signaling cascades where the G protein first becomes activated within a hormone-receptor-G protein complex and then the activated G protein dissociates from this receptor and seeks out its target/effector molecule. As will be outlined in sub-section 3., below, we in fact have obtained evidence that receptor heterodimer formation is transient and that following trans-phosphorylation events, the receptor dimer dissociates into its component monomeric receptors.

2. Interactions of the EGF receptor with other members of subclass 1- It seemed interesting that the signaling cascade involving ErbB3 and p85 in PC12 cells (described in the preceding section) appeared to be specifically initiated by heregulin and not by EGF. Both heregulin and EGF stimulated the tyrosine phosphorylation of ErbB3 with the stimulatory effects of heregulin, but not EGF, being greatly enhanced in PC12 cells that over-expressed Neu/ErbB2. The electrophoretic mobilities of the ErbB3 bands were clearly different for heregulin and EGF treatment (**see Gamett et al., 1995; Appendix I**). The heregulin-stimulated PC12 cells showed the two forms of ErbB3 described in the preceding section. The faster mobility ErbB3 phospho-band, stimulated by heregulin treatment, co-migrated with the slower mobility EGF-stimulated ErbB3 phospho-band. Thus, both the EGF receptor and Neu/ErbB2 were able to elicit a common phosphorylation event within the ErbB3 protein and this phosphorylated ErbB3 species (when heregulin-stimulated) remained complexed with Neu/ErbB2. There also was an ErbB3 phospho-band detected in EGF-treated PC12 cells that had a greater mobility than any of the phospho-bands detected in heregulin-treated cells. While the addition of EGF to PC12 cells over-expressing Neu/ErbB2 led both to the tyrosine phosphorylation of Neu/ErbB2 and ErbB3, neither of these phosphorylated proteins appeared to form a stable complex with p85.

An important question concerns the functional outcome of EGF receptor-Neu/ErbB2 interactions. Various studies have reported that the EGF receptor and Neu/ErbB2 will physically associate within cells and that this complex formation leads to trans-phosphorylation (28,29). However, the functional consequence of this interaction has not been determined. We used a well

defined *in vitro* system to try to determine the functional outcome of the trans-phosphorylation of Neu/ErbB2 by the EGF receptor. Purified plasma membranes from human epidermoid carcinoma (A431) cells were used as a source of EGF receptor because this preparation was highly active (when assayed for tyrosine kinase activity) and showed a strong tyrosine phosphorylation of the EGF receptor. The Neu/ErbB2 protein used in these experiments was an insect cell-expressed 85 kDa soluble Neu/ErbB2 tyrosine kinase domain that contained the entire intracellular portion of the rat Neu/ErbB2 receptor except for 12 amino acid residues in the juxtamembrane region (this is designated from here on as NTK). The NTK was partially purified from the soluble fraction of recombinant virus-infected Sf9 cells by gel filtration on a Bio Gel A-0.5 column and was the only detectable phospho-protein in the preparation after the addition of ATP (19).

NTK, alone, was capable of an autophosphorylation reaction that yielded a maximum stoichiometry of incorporation of ~1 mol phosphate per mol NTK. In the presence of the EGF-stimulated EGF receptor, the final extent of tyrosine phosphorylation of NTK was doubled (to ~2 mol $^{32}P_i$ incorporated per mol of NTK). These results suggested that the EGF receptor phosphorylated NTK at a residue that was inaccessible to autophosphorylation. We then found that under conditions where the autophosphorylation of NTK was weak and barely detectable, while the EGF receptor-catalyzed tyrosine phosphorylation of NTK was strong, there was no significant difference in the ability of NTK to phosphorylate either a tyrosine-containing peptide (i.e. E_4Y_1, a repeating polymer of glutamic acid and tyrosine) nor a protein phospho-substrate, the 85 kDa regulatory subunit (p85) of the PI 3-kinase. These results at first suggested that the EGF receptor-catalyzed trans-phosphorylation of Neu/ErbB2 did not stimulate Neu/ErbB2 kinase activity toward exogenous substrates (however, see below).

One clear outcome of the EGF receptor-catalyzed trans-phosphorylation of NTK was an enhanced ability of NTK to bind SH2-domain containing proteins (i.e. Src and p85). The enhanced binding of NTK to a GST-p85 protein was not translated into an enhanced ability of NTK to phosphorylate p85. Thus, it is possible that the enhancement in the binding of NTK to p85 has another purpose, i.e. to recruit p85 (or a related SH2 domain-containing protein) to the membrane.

It was interesting that this type of signaling mechanism did not appear to be bi-directional. Specifically, we did not detect any ability of the recombinant Neu/ErbB2 tyrosine kinase domain to phosphorylate the EGF receptor nor to significantly promote the ability of the EGF receptor to associate with an SH2 domain-containing protein, i.e. p85. Moreover, we have not detected a heregulin-stimulated phosporylation of the EGF receptor in rat PC12 cells under conditions where an EGF-stimulated tyrosine phosphorylation of Neu/ErbB2 was observed (Gamett and Cerione, data not shown).

Recently, we have obtained data suggesting that the formation of an EGF receptor-Neu/ErbB2 complex in PC12 cells does result in the activation of the Neu/ErbB2 tyrosine kinase. These results differed from those obtained using the *in vitro* system to characterize EGF receptor-Neu/ErbB2 interactions (described above). Specifically, we have found that when Neu/ErbB2 was precipitated from EGF-treated cells, it had a higher tyrosine kinase activity toward the synthetic peptide substrate, E_4Y_1, compared to Neu/ErbB2 precipitated from non-EGF-treated cells. These results then suggest either that the EGF receptor promotes the interaction of another tyrosine kinase (e.g. Src) with Neu/ErbB2, which then stimulates Neu/ErbB2 activation, or that the EGF-stimulated formation of a heterodimer between the full length EGF receptor and full length Neu/ErbB2 causes the activation of Neu/ErbB2 tyrosine kinase activity. We do not favor the former possibility because we have not been able to demonstrate that c-Src binds Neu/ErbB2. However, the possibility that the formation of a heterodimer between the full length EGF receptor and Neu/ErbB2 tyrosine kinase leads to the trans-phosphorylation and activation of Neu/ErbB2 would have interesting implications because it would suggest that growth factor binding to different receptors (i.e. EGF binding to the EGF receptor or heregulin binding to ErbB3) could promote the formation of heterodimers that contain Neu/ErbB2 and result in its activation. It also has interesting implications in light of the findings summarized in the next section.

3. Growth factor-stimulated receptor-receptor interactions are more complex than originally imagined- The finding that the tyrosine phosphorylated ErbB3 protein, but not Neu/ErbB2, formed a tight complex with the 85 kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase, described in Section 1. (above), was rather surprising. This suggested that the heregulin-stimulated ErbB3-Neu/ErbB2 heterodimer represented a transient complex rather than a stable complex, such that following the trans-phosphorylation of ErbB3, it dissociated from Neu/ErbB2 and was able to bind to cellular target/effector molecules.

During the past year, we have discovered that the mechanisms underlying growth factor-stimulated heterodimer formation are more complicated than originally suspected, but nonetheless pose some novel and interesting implications regarding growth factor-coupled signaling (**Gamett et al., 1997; Appendix I**). This discovery began with the finding that treatment of PC12 cells with EGF resulted in the precipitation of ErbB3 when using a specific anti-Neu antibody (Figure 5). This was similar to what we had observed following treatment of the cells with heregulin; however, the important difference was that EGF did not bind to either Neu/ErbB2 or ErbB3. Thus, the ErbB3-Neu/ErbB2 complex that formed in response to EGF treatment may be the outcome of a secondary receptor dimer. The idea was that EGF first stimulates the formation of an EGF receptor-Neu/ErbB2 heterodimer but after EGF-stimulated trans-phosphorylation of Neu/ErbB2, it dissociates from the EGF receptor and undergoes a new interaction with ErbB3.

In a separate set of experiments, we found that when PC12 cells were first stimulated by heregulin, and then Neu/ErbB2 was immunoprecipitated with a specific anti-Neu/ErbB2 monoclonal antibody and the tyrosine kinase activity in the resuspended pellet was assayed in the presence of the tyrphostins AG879 (a specific Neu/ErbB2 inhibitor) and AG556 (a specific EGF receptor inhibitor) (30), the tyrosine kinase activity was not only inhibited by AG879 but also by AG556. This suggested that another protein tyrosine kinase (e.g. the EGF receptor) co-precipitated with Neu/ErbB2 in a heregulin-dependent manner. We then verified this through Western blot analyses, where we examined anti-Neu/ErbB2 immunoprecipitates for the presence of the EGF receptor. We found that when the experiment was performed in the presence of the EGF receptor-specific tyrphostin, AG1478, a strong EGF receptor band was found in the Neu/ErbB2 immunoprecipitates. On the other hand, we have found that inhibition of Neu/ErbB2 tyrosine kinase activity by the specific Neu/ErbB2 antagonist AG879, significantly reduced the presence of the EGF receptor in Neu/ErbB2 immunoprecipitates.

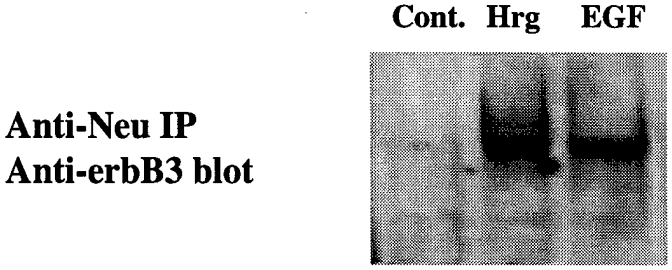


Figure 5: EGF induces the association of Neu/ErbB2 with ErbB3. PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. The cells were then stimulated for 2 minutes with either heregulin (Hrg) or EGF (20 nM or 100 ng/ml, respectively). The cells were then lysed and subjected to immunoprecipitation using anti-Neu/ErbB2 antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminiscence. Data was taken from Ganmett et al., 1997; Appendix.

These results appear to be consistent with the following scheme presented in Figure 6 (also see **Gamett et al., 1997; Appendix I**). The addition of heregulin (HRG) to cells stimulates the formation of receptor heterodimers between Neu/ErbB2 and either ErbB3 (as shown in Figure 5, lane 2) or between Neu/ErbB2 and ErbB4. In the former case, the binding of heregulin to Neu/ErbB2 within the ternary complex stimulates Neu/ErbB2 tyrosine kinase activity, leading to

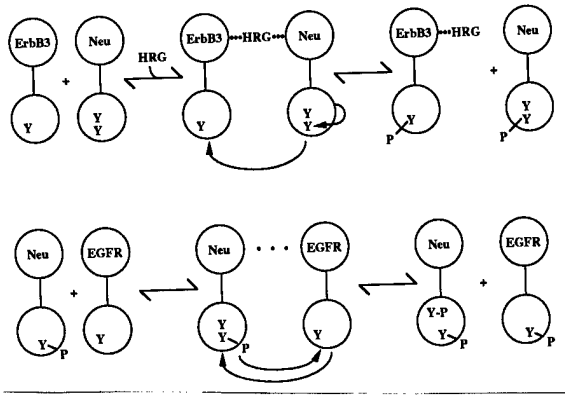


Figure 6: Depiction of heregulin-stimulated EGF receptor-Neu/ErbB2 secondary dimer formation. Heregulin first stimulates the formation of a Neu/ErbB2-ErbB3 (primary) heterodimer. Trans-phosphorylation of ErbB3 results in its dissociation from an activated Neu/ErbB2 receptor. Neu/ErbB2 then forms a (secondary) heterodimer with the EGF receptor. Trans-phosphorylation can then result in the dissociation of this secondary receptor heterodimer complex.

increased autophosphorylation and trans-phosphorylation of ErbB3. Since ErbB3 shows no detectable tyrosine kinase activity, there is no trans-phosphorylation (by ErbB3) of Neu/ErbB2. The phosphorylation of ErbB3 then results in its dissociation from Neu/ErbB2, such that ErbB3 is free to associate with p85 as we initially observed (**Gamett et al., 1995; Appendix I**). However, the autophosphorylated Neu/ErbB2 is now activated and able to bind to other (non-phosphorylated, inactive) receptors, in particular the EGF receptor. This then results in the formation of a secondary dimer between Neu/ErbB2 and the EGF receptor and further trans-phosphorylation events. The trans-phosphorylation of Neu/ErbB2 by the EGF receptor would presumably result in the dissociation of this secondary dimer, such that Neu/ErbB2 and the EGF receptor would then be free to interact with specific cellular phospho-substrates. Thus, we find that the inhibition of EGF receptor tyrosine kinase activity stabilizes the formation of the secondary dimer and thereby increases the amount of EGF receptor detected in Neu/ErbB2 immunoprecipitates. However, the inhibition of Neu/ErbB2 tyrosine kinase activity would prevent the dissociation of the (heregulin-stimulated) primary dimer between Neu/ErbB2 and ErbB3 (or ErbB4) and thereby inhibit secondary dimer formation.

Overall, these findings now suggest that growth factor-stimulated dimer formation between members of the EGF receptor family is analogous to a typical enzyme-substrate interaction, where one monomeric receptor tyrosine kinase acts as an enzyme, catalyzing the trans-phosphorylation of the partner monomeric receptor (the substrate). Once the receptor serving as a substrate is phosphorylated, it becomes the product of the enzyme reaction and dissociates from the receptor kinase (i.e. the enzyme). Such a mechanism raises a number of important implications. First, the trans-phosphorylation of a receptor such as Neu/ErbB2 within the primary dimer represents an activation event, such that Neu/ErbB2 is able to seek out other receptors and phosphorylate them in a ligand-independent manner. This would be consistent with our findings (preceding section) that EGF addition to cells appeared to result in the activation of Neu/ErbB2 tyrosine kinase activity (i.e. as an outcome of EGF-stimulated EGF receptor-Neu/ErbB2 primary dimer formation). Secondly, this mechanism would now suggest that growth factor-coupled signaling in fact occurs as 'waves of signaling events'. The first wave would represent the interactions of cellular phospho-substrates with monomeric, phosphorylated receptors that arise from the primary receptor dimerization event, and then a second wave of signaling events would follow secondary dimer formation. Because Neu/ErbB2-ErbB3 heterodimers can either form as primary dimers (in a heregulin-dependent fashion) or as secondary dimers (following EGF-stimulated EGF receptor-Neu/ErbB2 primary dimer formation and potentially following heregulin-stimulated ErbB4-Neu/ErbB2 primary dimer formation), it will be important to identify the cellular phospho-substrates that bind to phosphorylated ErbB3 for each of these different cases. For example, does p85 only bind ErbB3 as an outcome of the heregulin-stimulated Neu/ErbB2-ErbB3 primary dimer

but not following the formation of a secondary dimer between Neu/ErbB2 and ErbB3? It also will be important to determine if Neu/ErbB2 is essential for primary and secondary dimer formation (i.e. is it a unique property of Neu/ErbB2 that allows these events to occur) and do human breast cancer cells and dog breast cancer cells show similar dimerization events as detected in PC12 cells?

4. Identification of a specific EGF-stimulated phospho-substrate in human breast cancer cells as the Cbl proto-oncogene product- In the original proposal submitted to the DOD, our plan was to first understand the regulation of Neu/ErbB2 tyrosine kinase activity by heregulin and EGF in intact cells and then begin to identify signaling activities that were activated downstream from receptor tyrosine kinases. The initial idea was to examine ErbB3-Src interactions, however, as indicated above, our experiments failed to show any evidence for such interactions in cells. Given these negative results, we then decided to cast a broader net and search for cellular (cytosolic) proteins whose phosphorylation was strongly stimulated by EGF or by heregulin in human breast cancer cells. While performing such a screen in MDA-MB-468 human mammary carcinoma cell lysates, we found a 130 kDa protein (originally designated p130) that became highly tyrosine phosphorylated in an EGF-dependent manner and bound to GST-Src-SH3 and GST-Src-SH2 fusion proteins (Figure 7, below). We subsequently found that p130 also bound effectively to the amino-terminal SH2 domain of the adaptor molecule, Grb2. In order to determine the identity of p130, we probed blots with antibodies against several 120-130 kDa proteins. Two 130 kDa proteins that were of particular interest were c-Cbl and c-Cas (31,32);

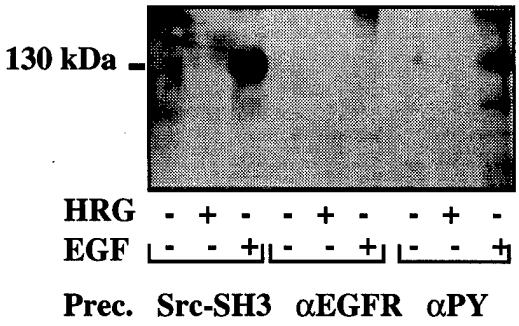


Figure 7: Phosphorylation of an 130 kDa protein stimulated by EGF in human breast cancer cells. MDA-MB-468 cells were serum-starved for 24 h, treated with EGF or with no growth factor and then lysed. The lysates were incubated with GST-Src-SH3 and glutathione-agarose beads, or with anti-EGF receptor antibody, or with anti-phosphotyrosine antibody. After precipitation, the pellets were resuspended and subjected to SDS-PAGE and Western blotted with an anti-phosphotyrosine antibody.

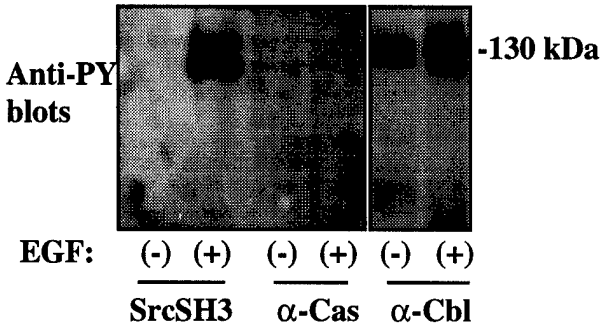


Figure 8: c-Cbl is the 130 kDa protein that is phosphorylated in response to EGF. MDA-MB-468 cells were treated as described in Figure 7. Lysates were then incubated with anti-Cas antibody, or with GST Src-SH3 fusion proteins, or with anti-Cbl and then the precipitated proteins were subjected to SDS-PAGE and Western blotted with anti-phosphotyrosine antibody.

c-Cbl was of interest because it has been found to be heavily tyrosine phosphorylated in hematopoietic cells in response to cytokines while Cas was of interest because it is a major phospho-substrate for the Src tyrosine kinase. We found that both c-Cbl and c-Cas could be precipitated from MDA-MB-468 cell lysates with both GST-Src-SH2 and GST-Src-SH3 fusion proteins; however, only the c-Cbl protein bound to GST-Grb2-SH3 and was tyrosine phosphorylated after EGF stimulation (see Figure 8 which shows that when Cbl was immunoprecipitated with a specific anti-Cbl antibody it was phosphorylated on tyrosine residues in an EGF-dependent manner). In addition, unlike Cas, c-Cbl was not detected to co-immunoprecipitate with anti-Src antibody. Thus, we have concluded that p130 is the Cbl proto-oncogene product.

We have found that EGF stimulates the tyrosine phosphorylation of c-Cbl in another breast cancer cell line, SKBR3, which over-expresses the EGF receptor, Neu/ErbB2 and ErbB3.

However, MDA-MB-453 human mammary carcinoma cells, which do not over-express the EGF receptor, do not show an EGF-stimulated tyrosine phosphorylation of c-Cbl. We also have found that heregulin does not stimulate the tyrosine phosphorylation of Cbl in any human mammary carcinoma cell that we have thus far examined (despite the fact that Neu/ErbB2 and ErbB3 are over-expressed in some of these cells). Taken together, these results indicate that the tyrosine phosphorylation of c-Cbl is a specific outcome of EGF receptor activation and occurs in breast cancer cells where the EGF receptor is over-expressed.

The specific mechanism underlying the coupling of Cbl to the activated EGF receptor in breast cancer cells is still being established. One likely possibility was that the interaction between the EGF receptor and c-Cbl was mediated by the binding of Cbl to the Grb2 adaptor molecule. The amino-terminal SH3 domain of Grb2 associates with Cbl, while the SH2 domain of Grb2 binds to a phospho-tyrosine residue within the carboxyl terminus of the EGF receptor. Thus, an EGF-stimulated activation of the EGF receptor could recruit a Grb2-Cbl complex, resulting in the phosphorylation of Cbl. However, we have recently found that Neu/ErbB2 can also be precipitated from human breast cancer cells (e.g. SKBR3 cells) with Grb2; however we do not find any evidence for a heregulin-stimulated association between Neu/ErbB2 and c-Cbl nor for a heregulin-stimulated phosphorylation of c-Cbl. Thus, it now seems more likely that it is a phospho-tyrosine binding (PTB) domain within the amino terminal region of c-Cbl that is responsible for its direct binding to tyrosine phosphorylated EGF receptor (probably in a manner analogous to the binding of the IRS proteins to the insulin receptor).

Another important question concerns the nature of the signaling pathway that is initiated by the EGF-stimulated tyrosine phosphorylation of Cbl in human breast cancer cells. Because during our initial investigations of p130 we suspected that this protein might be the c-Src substrate, Cas, and because Cas has been shown to form a complex with the Crk proto-oncogene product, we examined whether p130 might be capable of binding to Crk. We found that this in fact was the case, with an anti-Crk antibody co-precipitating the Crk and Cbl proteins in an EGF-dependent manner (Flanders et al., data not shown). The Crk proto-oncogene product is a 40 kDa cytoplasmic adapter protein which contains an amino terminal SH2 domain followed by two SH3 domains (33,34). Our preliminary evidence indicates that the SH2 domain of Crk binds to the tyrosine phosphorylated Cbl protein. However, it is interesting that we find that the EGF-dependent Cbl-Crk interaction in human breast cancer cells might be mediated by a prior association of c-Crk with the EGF receptor. To our knowledge, there have been no previous reports of *in vivo* binding of c-Crk to the EGF receptor. It is possible that the EGF receptor-Crk association is a specific characteristic of the MD-MB-468 breast cancer cells, since neither constitutive binding of c-Crk to the EGF receptor, nor EGF-stimulated tyrosine phosphorylation of c-Crk was detected in human epidermoid carcinoma (A431) cells. Thus, if simply high levels of EGF receptor were necessary to provide detectable EGF receptor-Crk complexes, we would have expected little difference between the MD-MB-468 cells and A431 cells. However, it is possible that the levels of expression of Crk are high in the breast cancer cells and thus give rise to a constitutive association between Crk and the EGF receptor.

We find that the time course for the EGF-stimulated phosphorylation of Cbl appears to correlate with the interaction between Cbl and c-Crk. A key question for the future concerns the signaling implications for the formation of an EGF-stimulated Cbl-Crk complex in human breast cancer cells. One possibility is that Cbl acts to interface Crk and Crk-binding proteins (e.g. the Abl tyrosine kinase) with the EGF receptor in mammalian epithelial cells. This possibility is particularly intriguing given that it has been proposed that aberrant tyrosine phosphorylation of Cbl by Abl may contribute to its transforming activity (35) and that transformation by oncogenic Crk (i.e. following the deletion of its carboxyl-terminal SH3 domain) may be due to the excess tyrosine phosphorylation mediated by the interaction between oncogenic Crk and Abl (36). However, it also is possible that Cbl plays some type of negative-regulatory role in the actions of the EGF receptor, based on the findings that the homolog for c-Cbl in *C. elegans*, the Sli-1 protein, has been reported to antagonize EGF-stimulated signaling (36a). For example, EGF-stimulated phosphorylation of Cbl may enable it to interact with other signaling molecules that normally bind to the EGF receptor (i.e. EGF-stimulated phosphorylation of Cbl may allow it to interact with Crk

and prevent Crk from binding to its normal signaling partners). It also has been shown that Cbl interacts with p85 in an EGF-dependent manner (37,38) and it has been suggested that Cbl may serve as an interface or an adaptor molecule for EGF receptor effects on PI 3-kinase activity in human epidermoid carcinoma (A431) cells (39). Thus, Cbl-Crk interactions may compete with Cbl-p85 interactions and thus serve to inhibit a specific (Cbl)-PI 3-kinase-mediated signaling pathway. Finally, recent work from our laboratory points to a potentially novel mechanism by which c-Cbl interferes with signaling to the nucleus (see section 6a., below).

5. Establish receptor tyrosine kinase signaling in dog mammary cells:Cbl is a specific EGF receptor phospho-substrate in dog mammary carcinoma cells- The ultimate goal of our research is to obtain information about receptor tyrosine kinase-initiated signaling pathways that can be used to design new strategies for screening and for interfering with the development of mammary carcinomas. Mammary neoplasms are the most common tumors of the female dog and about 40-50% of these mammary tumors are considered malignant (40,41). It has recently been argued that dogs may serve as an important model for human cancer, having a much greater similarity to humans compared to other commonly used experimentally induced tumor models (42). As a first step toward further validating the potential use of the dog as such a model, we set out to determine if common signaling activities could be detected in dog breast cancers. Given that the EGF-stimulated tyrosine phosphorylation of the c-Cbl protein, and its subsequent association with c-Crk, were specific and readily identifiable features of human breast cancer cells that highly express the EGF receptor, we have used these read-outs to compare the similarities between human and dog mammary carcinomas. We find that the dog mammary carcinoma cell line (CMT12) which over-expresses the EGF receptor yields a strongly (EGF-stimulated) phosphorylated protein at ~130 kDa with essentially identical mobility to p130 in human breast cancer cells (MD-MB-468) and in human A431 cells. We also have found that the 130 kDa tyrosine phosphorylated protein, when immunoprecipitated from CMT12 cells with an

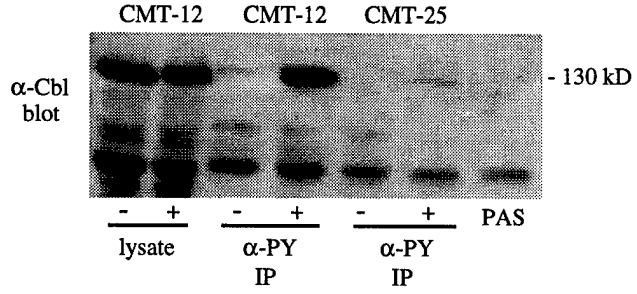


Figure 9: EGF-stimulated phosphorylation of c-Cbl in dog mammary carcinoma cells. Dog mammary carcinoma cell lines CMT-12 (which over-express EGF receptor) and CMT-25 (which do not over-express the EGF receptor) were stimulated with 100 ng/ml EGF (+) for 30 minutes or were incubated with buffer control (-). The cells were lysed and then immunoprecipitated with anti-phosphotyrosine antibody (αPY) and then Western blotted with anti-Cbl antibody.

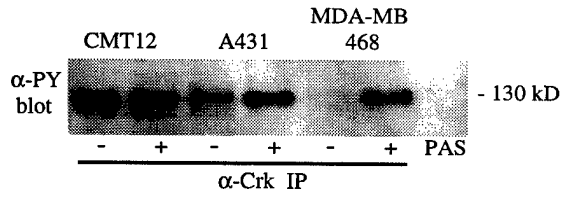


Figure 10: Binding of c-Cbl to c-Crk in dog mammary carcinoma cells. The dog cell line CMT-12, human epidermoid carcinoma (A431) cells, and human breast cancer cells (MDA-MB-468), which all over-express the EGF receptor, were treated with or without EGF as in Figure 9. The cells were then lysed, and c-Cbl was immunoprecipitated with anti-Crk antibody (αCrk) and Western blotted with anti-phosphotyrosine antibody.

anti-phosphotyrosine antibody, can be Western blotted with an anti-Cbl antibody (Figure 9; PAS represents a control lane where protein-A Sepharose was used alone). However, another dog mammary carcinoma cell line, CMT25, which does not over-express the EGF receptor does not show an EGF-stimulated tyrosine phosphorylation of the c-Cbl protein, just as is the case for the human MD-MB-453 cells which do not express the EGF receptor. We also have found that c-Crk is tyrosine phosphorylated in an EGF-dependent manner in dog mammary carcinomas and that an anti-Crk antibody will co-precipitate Crk and Cbl in an EGF-dependent manner, just as is observed in human A431 cells and in human breast cancer (MDA-MB-468) cells (Figure 10). Based on

these initial results, we are encouraged that the signaling pathways that are operating in dog mammary carcinomas that over-express the EGF receptor are similar to those operating in human breast cancer cells. We are now in the process of performing a detailed comparison between human and dog breast cancer cells with regard to a specific heregulin-stimulated signaling activity in the nucleus, which we have recently discovered (described in Section 6b., below).

6. New developments that came from the DOD funding: In the first 5 sections presented above, we describe the studies that were conducted to address our initial goals of understanding the regulation of Neu/ErbB2 in cells and signaling pathways that emerge from receptor tyrosine kinase activation in breast cancer cells. Below, we will describe some exciting new developments that have emerged and were made possible by the DOD funding.

a. Further connections between growth factor signaling and Rho-like GTP-binding proteins- One interesting possibility is that c-Cbl may play a role in signaling pathways that regulate the guanine nucleotide binding activity of small G proteins. In activated Jurkat T cells, the c-Crk related adaptor protein, CrkL, becomes associated with tyrosine phosphorylated c-Cbl (43). CrkL was constitutively associated with the guanine nucleotide exchange factor, C3G, suggesting a possible mechanism by which Cbl may influence the regulation of Ras and/or Ras-subfamily proteins. Moreover, recently we have found that members of a novel subgroup of the Dbl family of Rho-guanine nucleotide exchange factors, which are refered to as the Cool (for cloned-out-of a library) (**Bagrodia et al., in press, Appendix I**) molecules, bind to the c-Cbl homolog, Cbl-b (44). We originally identified the Cool molecules through their ability to bind to a serine/threonine kinase called PAK (for p21-activated serine/threonine kinase), which is a target for the Cdc42 and Rac GTP-binding proteins (45-47). The Dbl-homology domain found in all of the Cool family members would also predict that they bind Cdc42 or Rac. At present we have been studying three members of the Cool family, p50Cool-1 which binds and inhibits PAK, p85Cool-1 which competes with p50Cool-1 for binding PAK but does not inhibit PAK activity, and Cool-2 which binds and activates PAK. The mechanisms by which Cool-PAK interactions result in changes in PAK activity remain to be determined. The finding that p85Cool-1 binds to Cbl-b (Flanders et al., in preparation) provides a potentially interesting and previously unappreciated link between Cdc42/Rac signaling and receptor tyrosine kinase signaling. The Cbl-b protein was originally identified in human breast cancer cells and contains all of the same signaling motifs found in the c-Cbl protein. A proline-rich domain on Cbl-b binds to an SH3 domain on Cool. What is particularly interesting is that Cbl-b appears to bind p85Cool-1 with significantly higher affinity than does c-Cbl. We are now in the process of trying to establish the ramifications of a Cbl-Cool interaction. We have found that Cbl-b and PAK compete for binding to the Cool molecules. The ability of c-Cbl and Cbl-b to compete for binding to receptor tyrosine kinases would present the possibility for a rather intricate mechanism by which receptor tyrosine kinases can influence signaling via the small GTP-binding proteins Cdc42/Rac and their target, PAK (see Figure 11). We in fact have been searching for possible connections between Cdc42 and cell growth regulation because of the potential involvement of Cdc42 or related small GTP-binding proteins in Cbl-mediated signaling, as well as their possible involvement in a specific heregulin-stimulated pathway that culminates in the activation of a putative splicing factor (see Section 6b., below). An essential role for Cdc42 in cell growth regulation has been suggested by the finding that the Dbl oncoprotein is an upstream activator (i.e. a guanine nucleotide exchange factor) for Cdc42 (**see Zheng et al., 1996a,b**) and that other Dbl-related proteins, which cause cellular transformation, are nucleotide exchange factors for small GTP-binding proteins (**see Glaven et al., 1996**).

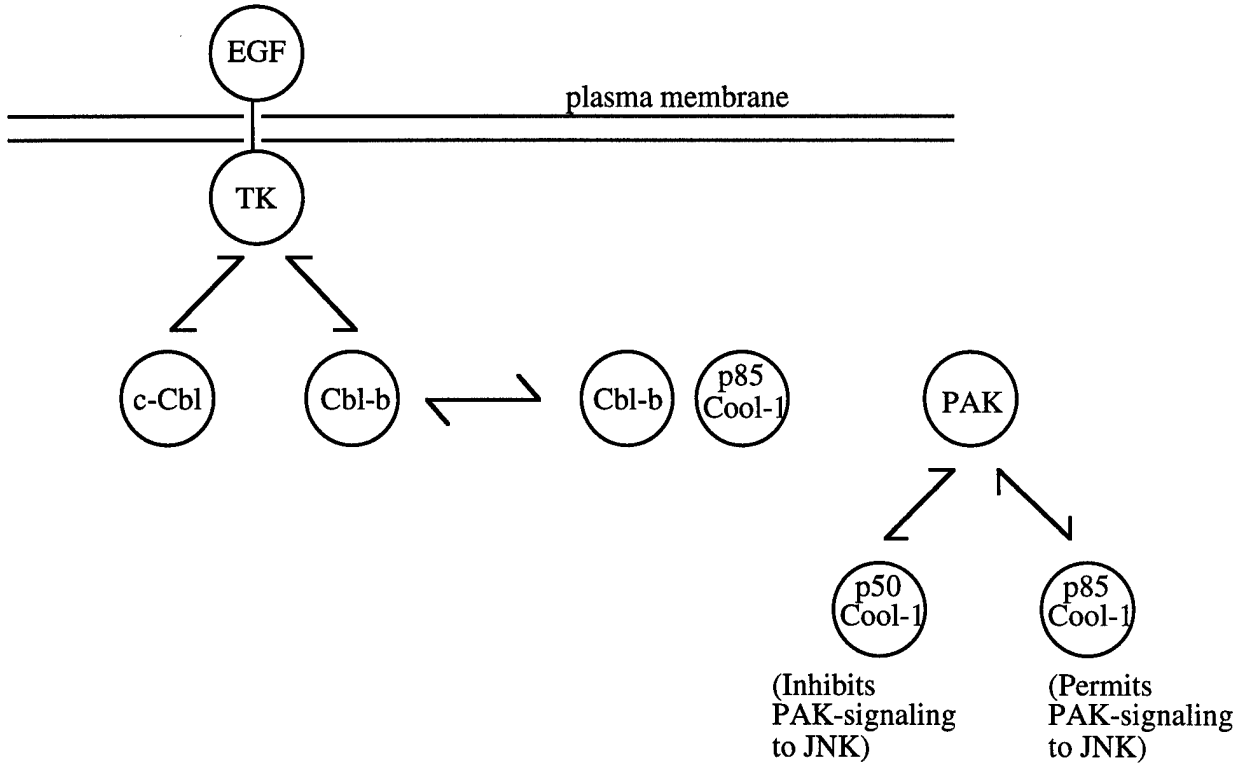


Figure 11: Schematic representation of the interactions of c-Cbl and Cbl-b with the EGF receptor tyrosine kinase and with p85Cool-1. It is suggested that c-Cbl and Cbl-b compete for binding to the EGF receptor. When c-Cbl binds to the receptor, Cbl-b is free to interact with p85Cool-1. This interaction prevents the binding of p85Cool-1 to the serine/threonine kinase, PAK. This in turn allows the binding of p50Cool-1 to PAK, resulting in an inhibition of PAK activity. Thus, c-Cbl, by binding to the EGF receptor, initiates a sequence of events that results in the inhibition of PAK signaling to the nuclear MAP kinase, the c-Jun kinase (JNK).

b. Identification of a novel heregulin-stimulated GTP-binding activity as the RNA splicing factor, CBP20- During the past year, we have been characterizing a GTP-binding activity that is present in nuclear extracts in every cell type that we have examined and is strongly stimulated by heregulin (**Wilson et al., submitted; Appendix**). We initially showed that in HeLa cells this activity (as assayed by the photo-incorporation of [α^{32}P]GTP) was most effectively stimulated by heregulin, whereas in rat PC12 cells, the activity was also strongly stimulated by nerve growth factor (NGF) (see Figure 12, page 23). This finding was consistent with earlier work from our laboratory (48), which indicated that heregulin, like NGF, was capable of stimulating neurite extension in PC12 cells. The heregulin/NGF-stimulated guanine nucleotide-binding activity corresponded to a protein of apparent M_r ~18 kDa, which we initially designated as p18. The incorporation of [α^{32}P]GTP into p18 was also catalyzed by the exposure of cells to ultraviolet radiation (Figure 13, page 24), suggesting a stress response similar to those observed with the nuclear MAP kinases, the c-Jun kinase (JNK1) and p38. The growth factor-stimulated binding of GTP to p18 also showed a cell-cycle dependence, such that it was strongest in G_1/S and not detected in G_0 nor when cells were blocked in mitosis (Figure 14, page 24). Perhaps most interesting, we have found that p18 activity is strongly activated in the human breast cancer cell line, SKBR3 (Figure 12A), even under conditions of serum-starvation. At the present time, we know very little about the detailed sequence of events that lead to the activation of p18. However, as will be elaborated upon below, this becomes an especially interesting issue because we have recently determined that p18 is identical to CBP20, an 18 kDa subunit of the nuclear RNA cap-binding protein complex [CBC (49)].

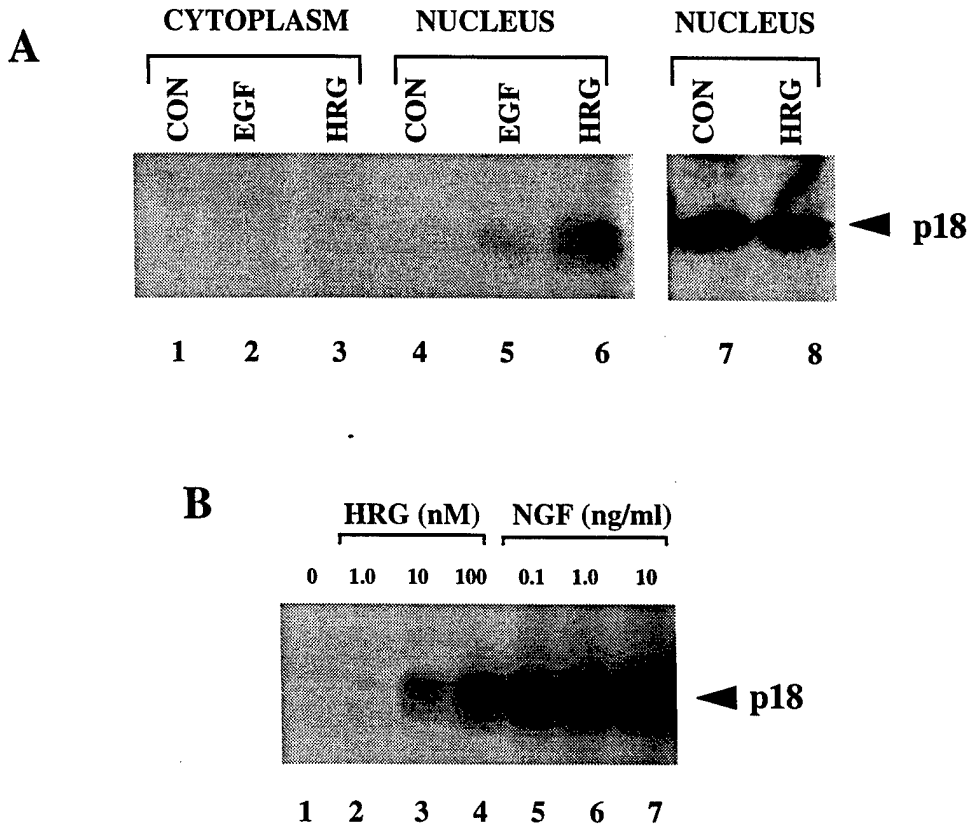


FIG. 12. **Growth factor sensitivity of an 18 kDa nuclear GTP-binding activity in HeLa cells, PC-12 cells, and the breast cancer cell line, SKBR3.** A) HeLa cells (*lanes 1-6*) were serum starved (*control lanes 1 and 4*) and then treated with 100 ng/ml EGF (*lanes 2,5*), or with 30 nM heregulin (HRG, *lanes 3,6*) for 15 minutes at 37°C. SKBR3 cells were serum starved (*lane 7*) and then treated with 1.0 nM heregulin for 1.5 hours (*lane 8*). The cells were then lysed and separated into cytoplasmic (*lanes 1-3, 7*), and whole nuclear (*lanes 4-6, 8*) fractions and assayed for GTP-binding using 45 µg of HeLa lysate or 50 µg of SKBR3 lysate. The samples were incubated for 10 minutes at room temperature with 20 µl of crosslinking buffer and 3 µCi [α^{32}P]GTP, followed by exposure to UV light for 15 minutes on ice. The samples were then separated by 15% SDS-PAGE, and the resulting gel was dried and exposed to X-ray film for 11 h to 3 days. B) A dose response experiment was performed using the addition of either heregulin (*lanes 2-4*) or NGF (*lanes 5-7*) to serum-starved PC-12 cells (*control, lane 1*) for 30 minutes at 37°C. For each dose of heregulin or NGF, 50 µg of total nuclear lysate protein were assayed for GTP-binding by UV-crosslinking with [α^{32}P]GTP, and then separated by 15% SDS-PAGE. The resulting gel was dried and exposed to X-ray film for 5-15 h.

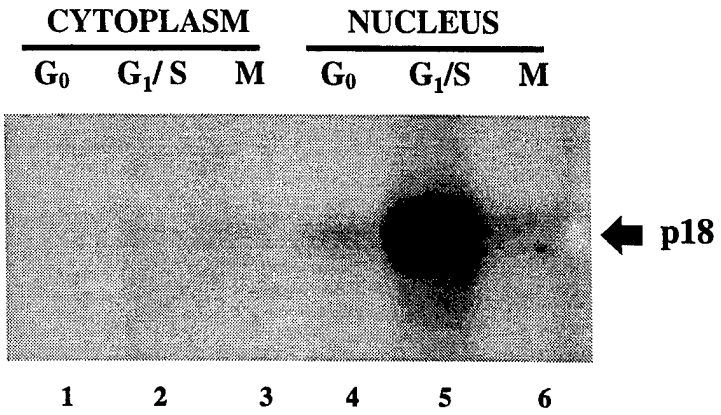


FIG. 13. **p18 is active to bind GTP in cells arrested in G_1/S phase of the cell cycle.** HeLa cells were arrested in G_0 by serum starvation (*lanes 1,4*), in G_1/S by 2.5 mM thymidine addition (*lanes 2,5*), and in M phase with 80 ng/ml nocodazole (*lanes 3,6*). Cells were then separated into cytoplasmic (*lanes 1,2,3*), and whole nuclear fractions (or a mitotic pellet was prepared for M phase arrest) (*lanes 4,5,6*), and then for each fraction, 50 µg of protein were assayed for [α^{32}P]GTP-binding activity by UV-crosslinking followed by 15% SDS-PAGE and autoradiography.

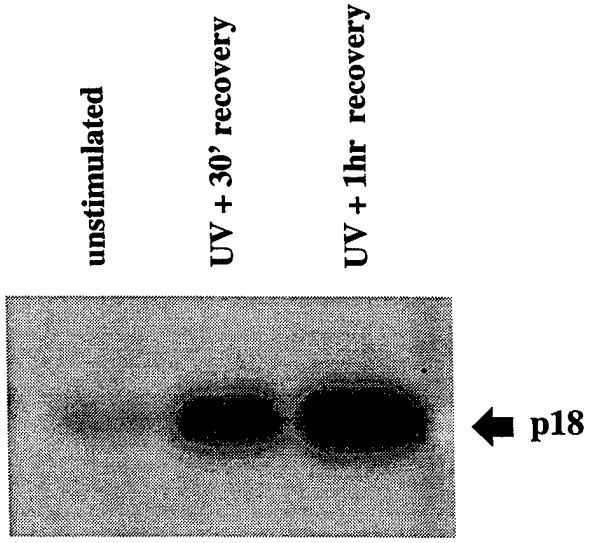
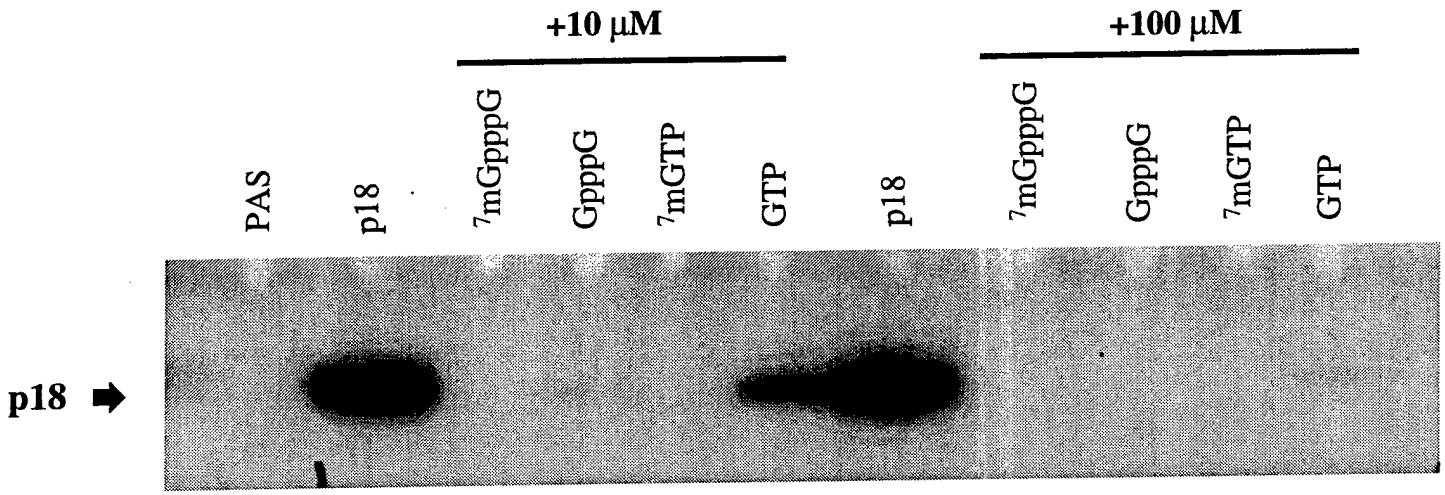


FIG. 14. **Exposure of cells to UV light stimulates the GTP-binding activity of p18.** PC-12 cells were serum-starved and then exposed to UV light for 2 minutes. Following exposure, cells were replenished with serum-free medium and allowed to recover for 30 minutes or one hour. Cells were then harvested, nuclear lysates were prepared, and 50 µg of nuclear lysate were assayed for [α^{32}P]GTP-binding to p18 by UV crosslinking followed by 15% SDS-PAGE and autoradiography.



IP: αCBP80
Assay: [α32P]GTP-binding

FIG. 15. **p18 [α32P]GTP-binding activity co-immunoprecipitates with CBP80 from PC-12 cells and is blocked by the addition of RNA cap-analogs.** Nuclear extracts were prepared from PC-12 cells growing asynchronously in culture. Five hundred micrograms of lysate were then immunoprecipitated with 5 μl of αCBP80 antiserum and 40 μl of protein A sepharose per lane. The immunoprecipitates were then washed four times with Tris-HCl, pH 7.4, 0.33% (v/v) Triton X-100, 133 mM KCl, 1 mM DTT, and 1 mM Na_3VO_4 and then resuspended in 30 μl of UV-crosslinking buffer. The immunoprecipitates were then assayed for [α32P]GTP-binding to p18 by UV crosslinking in the presence of 10 μM or 100 μM of the cap analogs 7mGpppG, GpppG, 7mGTP, or GTP. These proteins were then separated by 15% SDS-PAGE, and the resulting gel was dried and autoradiogrammed.

During our attempts to purify p18, we found that it eluted from gel filtration columns with an apparent size of ~150 kDa and appeared to co-purify with an ~80 kDa protein (designated p80), which we suspected might be essential for the GTP-binding activity of p18. The nuclear localization of p18 and its apparent requirement of p80 for binding guanine nucleotides were highly reminiscent of the biochemical properties of the RNA cap-binding complex (designated CBC), which has been implicated in precursor mRNA splicing and in RNA nuclear export (49,50). This complex is comprised of an 18 kDa protein (CBP20) and an 80 kDa protein (CBP80), which when associated are able to bind to a 5' cap structure that consists of a guanosine residue methylated at the N7 position and joined to the first encoded nucleotide of RNA. The first experimental indication that p18 and p80 may correspond to CBP20 and CBP80, respectively, came from Western blot analyses of the purified fractions of p18, which showed that these fractions were highly enriched in both the CBP20 and CBP80 proteins. We then found that p18 was capable of binding RNA-cap analogs even more effectively than GTP and that p18/GTP-binding activity could be immunoprecipitated from cells using an anti-CBP80 antibody (Figure 15, page 25). We also have found that *E. coli*-expressed CBP20 can be radiolabeled with [α^{32}P]GTP in a CBP80-dependent manner, and we have demonstrated serum-starved GTP-binding to recombinant CBP20 that was expressed in BHK21 cells (see **Wilson et al., Appendix**). Based on these findings, we have concluded that p18 is identical to CBP20.

We have gone on to show that heregulin will also stimulate the binding of p18/CBP20 to capped RNA and that it will cause a marked stimulation (~4 fold) of cap-dependent splicing using an *in vitro* assay system as described in (49). The stimulation by heregulin of CBP20 binding to RNA cap structures may have important implications regarding a previously unappreciated connection between receptor tyrosine kinase (i.e. Neu/ErbB2) signaling and RNA splicing, RNA export and/or protein synthesis. It also raises the possibility of some potentially interesting growth factor-regulated interactions between CBP20 and other proteins that have been implicated in RNA metabolism. Of particular interest is the GTP-binding protein Ran, which has been implicated in RNA trafficking and cell-cycle control, and its activator [guanine nucleotide exchange factor (GEF)] RCC1 (51,52). Studies with tsBN2 cells which possess a temperature-sensitive RCC1 protein have shown that the loss of RCC1 at the non-permissive temperature is accompanied by a marked increase in the ability of CBP20 to be labeled with [α^{32}P]GTP (Wilson and Cerione, unpublished findings). One possible explanation for these findings is that RCC1 serves as a guanine nucleotide-dissociation inhibitor (GDI) for CBP20, such that when CBP20 is associated with RCC1, GDP (or GTP) dissociation from CBP20 is blocked and the incorporation of radiolabeled GTP is inhibited. Thus, a growth factor (heregulin)-stimulated signaling pathway to the nucleus may in some manner disrupt the interaction between CBP20 and RCC1, thus enabling CBP20 to undergo guanine nucleotide exchange or the exchange of bound guanine nucleotide for capped RNA. We are now in the process of determining the specific nature of the signaling pathway that couples Neu/ErbB2 activation at the cell surface with the stimulation of CBP20 activity in the nucleus, with a particular emphasis being the possibility that Cdc42 or Rac signaling plays an important role in this pathway.

c. Identification of novel retinoic acid-induced signaling pathway- During our search for novel nuclear GTP-binding activities, we purified an 80 kDa protein from the nuclear pore which based on microsequencing appeared to be a member of a family of dual function GTP-binding protein/transglutaminases (53). Members of this family of proteins (designated from here on as TGases) appear to function as G protein signal transducers (54,55) and as Ca^{2+}-dependent enzymes which play potentially important roles in protein cross-linking and in regulating apoptosis, cell morphology, and cell adhesion (56,57). The TGase that we originally isolated from the nuclear pore/lamina fractions showed high GTP-binding activity relative to the cytosolic TGase, which was essentially inactive. This led us to find that the GTP-binding activity of the cytosolic TGase was tightly regulated by retinoic acid (RA), and that upon RA-stimulated activation, the TGase bound the eukaryotic initiation factor, eIF-5A (**Singh et al., 1998**). The eIF-5A protein has been suggested to participate in protein synthesis, either at the ribosomal subunit joining step and/or at a later stage of 80S ribosomal initiation complex formation (58). More recently, eIF-5A also has been suggested to play a role in RNA export, based on its ability to

interact with the HIV-1 protein, rev (59,60). Since eIF-5A is the only known protein to contain the amino acid hypusine, which is essential for eukaryotic cell viability, it has been argued to play a critical role in cell growth regulation. We have suggested that the interactions of the TGase with eIF-5A may be important for the movement of eIF-5A between the cytosol and the nucleus, during its involvement in different aspects of RNA processing. We recently have obtained data suggesting that while RA-stimulated TGase activity appears to be essential for RA-mediated protection against apoptosis, the over-expression of the TGase circumvents the tight regulation of TGase activity by RA and causes cell death (Singh et al., in preparation). It may be that over-expression of TGase, by persistantly binding eIF-5A, gives rise to an apoptotic outcome. Thus, we are now in the process of examining the levels of TGase in normal mammary epithelial cells and in human breast cancer cells and plan to determine whether heightened expression of TGase can kill breast cancer cells.

CONCLUSIONS

The EGF receptor, Neu/ErbB2 and other members of the subgroup 1 growth factor receptors have been the major focus of our studies funded by the DOD Breast Cancer initiative because the different members of this family have been implicated in human cancers. The amplification of the EGF receptor has been found in squamous cell carcinomas, bladder, lung, and liver cancers, and in gliomas (61,62). Over-expressed Neu/ErbB2 has been found in a significant proportion of human breast cancers (and hence its focus in these studies) (30-36) and ErbB3 also has been suggested to be associated with human malignancy (5).

Our initial intention was to understand how Neu/ErbB2 becomes activated by heregulin and then to determine the cellular consequences of Neu/ErbB2 activation. Overall, it is my feeling that we have been very successul in proving the underlying hypothesis that was the basis of our DOD proposal, i.e. that heregulin binds ErbB3 and activates Neu/ErbB2 as an outcome of ErbB3-Neu/ErbB2 heterodimer formation. In particular, we have demonstrated that heregulin stimulates the formation of ErbB3-Neu/ErbB2 complexes in intact cells and that this leads to the tyrosine phosphorylation of ErbB3. In PC12 cells, where heregulin stimulated signaling is coupled to neurite extension, we also found the formation of a specific interaction between tyrosine phosphorylated ErbB3 and the regulatory subunit of the PI 3-kinase activity. We hypothesize that this interaction represents an important early step in heregulin-coupled signal transduction. We also have shown that other types of receptor heterodimer complexes are possible, including EGF receptor-ErbB3 and EGF receptor-Neu/ErbB2 interactions; the latter appears to represent an alternative pathway (i.e. in addition to heregulin) for the activation of Neu/ErbB2. In addition, we have uncovered a new mechanistic aspect of receptor-receptor interactions and have shown that members of the EGF receptor family form heterodimers that are transient in nature. Thus, Neu/ErbB2-ErbB3 heterodimers can form as primary dimers, stimulated by the initial binding of heregulin to ErbB3, or as secondary dimers as an outcome of the dissociation of activated Neu/ErbB2 from EGF-promoted EGF receptor-Neu/ErbB2 primary dimers. Interestingly, it appears that the phosphorylation profiles for ErbB3 which arise from these two types of Neu-ErbB3 heterodimers are not identical. This then argues that the ability of different growth factors to stimulate the formation of both primary and secondary receptor dimer combinations adds to the diversity of signaling activities that can be generated by the EGF receptor family.

The number of possibilities that exist for receptor heterodimer combinations, and our recent finding that secondary dimerization events can occur, greatly complicates the picture when considering the possible biological activities that are likely to be triggered by each of the members of the EGF receptor family. However, we have obtained some very interesting leads over the past few years, which I believe hold exciting promise for the future. One involves the specific tyrosine phosphorylation of c-Cbl by the EGF receptor in breast cancer cells. This is a highly specific phosphorylation event (which cannot be stimulated by heregulin or through its activation of Neu/ErbB2) which appears to reflect a specific and direct interaction between the EGF receptor and c-Cbl. It has long been suspected that c-Cbl will act as an antagonist of EGF receptor signaling, based on the actions of its homolog, Sli-1, in *C. elegans*. Recently, we have obtained some

evidence that might provide a potential mechanism for such an effect. Specifically, we have found that the related protein, Cbl-b, which was first identified in human breast cancer cells, may provide an important interface between the EGF receptor and signaling that is mediated by the Ras-related protein Cdc42 (and/or the related G protein, Rac). We have shown that activators (guanine nucleotide exchange factors) for Cdc42 and related G proteins are often oncogenic and we recently have discovered a new family of putative adaptors/regulators (called the Cool molecules) for Cdc42 and the Cdc42-target, PAK. We have found that Cbl-b directly binds the Cool molecules and suspect that the competition between c-Cbl and Cbl-b for the EGF receptor tyrosine kinase may influence EGF receptor signaling to Cdc42 and PAK. We are now in the process of establishing the implications of the over-expression of Cbl-b, or the over-activation of Cdc42 or PAK in human breast cancer cells.

Another recently discovered lead regarding heregulin-coupled signaling to the nucelus involves an ~18 kDa protein which appears to be essential for the normal regulation of RNA splicing. We initially discovered this protein while searching for new types of nuclear GTP-binding activities; however, after purification efforts, we determined that this GTP-binding activity was identical to the CBP20 protein, which normally binds the ^{7}methyl guanosine cap structure of precursor RNA molecules that are transcribed by RNA polymerase II. Since CBP20 activity appears to be strongly regulated by heregulin, we now are positioned to obtain new information regarding how Neu/ErbB2 and/or related receptor tyrosine kinases influence different aspects of RNA metabolism. Because approximately 15% of all genetic diseases have been attributed to alterations in RNA splicing (70), and because there have been a number of correlations drawn between the generation of improper RNA transcripts and tumorigenesis (71,72), these findings hold exciting potential for providing some novel insights into how growth factors normally influence RNA processing at the level of gene expression and how a loss of this regulation may have important implications for the development of various diseased states including cancer.

Future studies will be directed at understanding how heregulin-promoted activation of CBP20 occurs (because it represents such a specific nuclear end-point for heregulin-coupled signaling) and why this activity appears to be constitutive in certain human breast cancer cells. We now believe that a highly sensitive criterion for evaluating the dog as an appropriate animal model for heregulin-signaling will be through comparisons of CBP20 activation in human and mammary canine mammary carcinomas. As we learn more about other nuclear proteins that bind to CBP20 in human breast cancer cells, we also will determine whether these interactions are no longer properly regulated in canine mammary carcinomas. Our plan is to begin these studies with canine mammary breast cancer cell lines that are already available in the laboratory (e.g. CMT12 cells which over-express the EGF receptor and CMT25 cells which over-express Neu/ErbB2). However, our intention is to extend these studies to other cell lines that we develop from mammary gland tumor tissues collected from dogs at the time of surgery here at the Veterinary Medical Center. Once we have established that CBP20 activity in canine mammary carcinomas is similar to what we find in human breast cancer cells, we then will assay nuclear lysates from canine tumor cells to determine whether RNA splicing activity is de-regulated. The longer term goal will be to identify reagents that attenuate CBP20 activation and aberrant splicing activity [e.g. tyrosine kinase inhibitors, RNA cap-analogs and/or small cell-permeable RNA-binding molecules similar that are being used to target specific DNA sequences (73)], with the ultimate hope of identifying promising compounds that might be useful in clinical trials on dogs that have been diagnosed with mammary carcinomas.

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Publications Arising from DOD Support

Gamett, D.C., Greene, T., Wagreich, A.R., Kim, H.-H., Koland, J.G., and Cerione, R.A. (1995) Heregulin-stimulated Signaling in Rat Pheochromocytoma Cells. Evidence for ErbB3 interactions with Neu/ErbB2 and p85. *J. Biol. Chem.* **270**, 19022-19027.

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Bagrodia, S., Taylor, S.J., Jordon, K.A., Van Aelst, L., and Cerione, R.A. A novel regulator of p21 activated kinases. *J. Biol. Chem.* In press.

Abstracts

Flanders, J.A., and Cerione, R.A.: EGF-dependent phosphorylation of an SH2/SH3-binding protein in mammary cancer cells. Eleventh Annual Meeting on Oncogenes. Frederick, Maryland, June 1995.

Flanders, J.A., Wilson, K., Singh, U.S., Gamett, D., and Cerione, R.: C-Cbl is a specific signaling partner for the EGF receptor. Department of Defense Breast Cancer Research Program: An Era of Hope. Washington, D.C., October 1997.

Wilson, K.F., Singh, U.S., and Cerione, R.A.: Identification of a growth factor stimulated, nuclear GTP-binding activity. FASEB Meeting, Summer 1995.

Wilson, K.F., and Cerione, R.A.: A growth factor sensitive, nuclear GTP-binding activity corresponds to the RNA cap-binding protein, CBP20. Keystone Meeting, February 1997.

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APPENDIX

Publications and Manuscripts Arising from DOD Support

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Heregulin-stimulated Signaling in Rat Pheochromocytoma Cells

EVIDENCE FOR ErbB3 INTERACTIONS WITH Neu/ErbB2 AND p85*

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We have reported that overexpression of Neu leads to heregulin-stimulated neurite outgrowth and the tyrosine-phosphorylation of Neu and other cellular proteins in PC12 cells. Considering that Neu/ErbB2 alone is not able to functionally couple to heregulin, we looked for the possible involvement of ErbB3 in these neurite outgrowth and tyrosine phosphorylation responses. We found that heregulin stimulates the tyrosine phosphorylation of endogenous ErbB3 protein in PC12 cells and that this phosphorylation, like that of Neu, is greatly enhanced in cells that overexpress Neu. Furthermore, overexpression of ErbB3 in PC12 cells led to heregulin-stimulated neurite extension. In addition to becoming tyrosine-phosphorylated, Neu/ErbB2 and ErbB3 associate with each other, and each associates with the 85-kDa regulatory subunit (p85) of phosphatidylinositol 3-kinase in a heregulin-dependent manner. Thus, Neu/ErbB2 and ErbB3 appear to cooperate to mediate the heregulin signal in PC12 cells. Like heregulin, epidermal growth factor (EGF) also stimulates the tyrosine phosphorylation of both Neu and ErbB3. However, there are clear differences between the EGF- and heregulin-stimulated phosphorylations of ErbB3. In the heregulin response, two tyrosine-phosphorylated forms of ErbB3 are detected. Of these, only the more quickly migrating form (on SDS-polyacrylamide gel electrophoresis) is found to be associated with Neu, whereas the other, more slowly migrating form is uniquely capable of forming stable complexes with p85. In the EGF response, at least two tyrosine-phosphorylated forms of ErbB3 are detected, but these phosphoproteins have distinctly lower apparent molecular weights compared with the heregulin-stimulated ErbB3 phosphoproteins and do not complex with p85. Thus the formation of a stable ErbB3-p85 complex in PC12 cells is a unique outcome of heregulin signaling that correlates with the differences in cell morphology induced by the activated EGF receptor and the Neu tyrosine kinase.

The subclass 1 receptor tyrosine kinases include the epidermal growth factor (EGF)[1] receptor, the Neu/ErbB2 tyrosine kinase, and the ErbB3 and ErbB4 proteins. This receptor family has received a great deal of attention because of the suspected involvement of different members of the family in the development of human cancers. This has especially been true for Neu/ErbB2. The rat Neu tyrosine kinase was first identified in rat neuroblastomas induced by the chemical mutagenesis of rat embryos (1). More recently, the human homolog of the rat Neu protein, commonly designated ErbB2 (or Her2), has been implicated in the development of human breast and cervical cancers (2).

It is interesting that a variety of studies also have implicated the involvement of receptor tyrosine kinases in developmental processes; the most well known examples being in the development of the compound eye in *Drosophila* (3) and in vulval development in *Caenorhabditis elegans* (4). In the case of Neu/ErbB2, it has been suspected that this tyrosine kinase may play a role in neuronal development, because a putative activator/growth factor for Neu/ErbB2, called heregulin (5) or the Neu differentiation factor (6), is identical to glial growth factor (7) and acetylcholine receptor-inducing activity (8). Along these lines, we recently have shown that the expression of a transforming version of the Neu tyrosine kinase (where the valine residue at position 664 within the transmembranal domain has been changed to a glutamic acid) is capable of stimulating neurite extension in rat pheochromocytoma (PC12) cells (9). Moreover, we have found that the addition of heregulin to PC12 cells that overexpress the normal Neu tyrosine kinase also elicits neurite extension. These results then highlight two important points. The first is that activation of the Neu tyrosine kinase, either by a point mutation or by the addition of its putative activating ligand, elicits a cellular morphology that is similar to that induced by the nerve growth factor receptor (*i.e.* the Trk tyrosine kinase) but distinct from the cellular effects elicited by the more similar EGF receptor. Thus, PC12 cells offer an excellent model system for distinguishing the specific features of the signaling pathways initiated by Neu/ErbB2 *versus* the EGF receptor.

The second major implication from the original studies of Neu effects in PC12 cells is the fact that the addition of heregulin leads to the apparent activation of the Neu/ErbB2 tyrosine kinase in these cells. This finding indicates that PC12 cells must contain the necessary components for the stimulatory regulation of Neu. This is an important point because we (10, 11) and others (12, 13) have shown that Neu/ErbB2 alone is not able to functionally couple to heregulin. Rather, it appears that the actual receptors for heregulin are the ErbB3 and ErbB4 proteins, suggesting that heregulin-stimulated heterodimer formation between Neu/ErbB2 and either ErbB3 or ErbB4 is necessary to stimulate Neu/ErbB2 tyrosine kinase activity. In the case of ErbB3, the need for coupling to Neu/ErbB2 is most clear, because ErbB3 by itself has little or no intrinsic tyrosine kinase activity (14).

In the present studies, we set out to expand upon these two

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[1] The abbreviation used is: EGF, epidermal growth factor.

implications. Specifically, we wanted to determine if the ErbB3 protein participated with Neu/ErbB2 in eliciting neurite extension in PC12 cells, and we set out to identify differences between the signaling cascades initiated by heregulin and those stimulated by EGF. We found that overexpression of either Neu/ErbB2 or ErbB3 renders PC12 cells biochemically and morphologically responsive to heregulin. Neu/ErbB2 and ErbB3 associate with each other, become tyrosine-phosphorylated, and associate with the p85 regulatory subunit of phosphatidylinositol 3-kinase after heregulin treatment. The events triggered by heregulin are distinguished from those initiated by EGF by the formation of a tyrosine-phosphorylated ErbB3 species with a retarded electrophoretic mobility and unique ability to complex with p85.

MATERIALS AND METHODS

Cells, Growth Factors, and Antibodies—PC12 cells (obtained from Dr. M. Chao, Cornell University Medical School) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. Anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories, anti-c-Neu antibody-3 was from Oncogene Science Inc. (Manhasset, NY), and anti-ErbB4 was from Santa Cruz Biotechnology. Anti-p85 antibody was a generous gift from Drs. L. Cantley and C. Carpenter (Harvard Medical School). Production and use of the 2F12 anti-ErbB3 monoclonal antibody (Neo Markers, Fremont, CA) have been described elsewhere (15). Recombinant heregulin (rHRG-$\beta1_{177-241}$) was provided by by Dr. Mark Sliwkowski, Genentech, Inc.

Transfections—The cDNA for bovine ErbB3 (10) was subcloned into pcDNAI/neo (Invitrogen) and introduced into PC12 cells using Lipofectin (Life Technologies, Inc.) according to the supplier's protocol. Stably transfected cells were selected in 400 μg/ml (active) antibiotic G418 (Geneticin, Life Technologies, Inc.).

Growth Factor Treatment, Immunoprecipitation, and Western Blot Analysis—Cells were grown to near confluence in Dulbecco's modified Eagle's medium plus 10% horse serum and 5% fetal bovine serum on 150-mm tissue culture dishes. 16–20 h prior to growth factor treatment, media were changed to Dulbecco's modified Eagle's medium with 0.1% fetal bovine serum. Cells were then removed from the dishes by trituration in Hanks' balanced salt solution without calcium or magnesium, pelleted (500 rpm for 5 min), and resuspended in Dulbecco's modified Eagle's medium without serum. Cells were dispensed at 1.0×10^7/1.5 ml/microfuge tube. Heregulin (20 nM) or EGF (100 ng/ml) was added, and the cells were incubated at 37 °C for 5 min. The treatment was ended by plunging the microfuge tubes containing the cells into ice followed immediately by centrifugation to pellet the cells and aspiration of the factor-containing supernatants. The cell pellets were then lysed in Tris-buffered saline with 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EGTA, 40 mM sodium fluoride, 100 μM ammonium molybdate, and 1 mM sodium orthovanadate. The lysates were incubated for 15 min on ice, after which insoluble materials were pelleted in a microfuge (10 min). Each supernatant was then incubated for 2 h on ice with 30 μl of a 50% suspension of protein A-Sepharose (Sigma) and the indicated antibodies. The immune complexes were pelleted, boiled in 40 μl of Laemmli sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis (8% acrylamide), and transferred to nitrocellulose. The blots were blocked in 3% bovine serum albumin in Tris-buffered saline plus 0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C in primary antibody as indicated. Blots were analyzed by ECL (Amersham).

RESULTS

Overexpression of the Rat Neu Tyrosine Kinase in PC12 Cells—The overexpression of the normal rat Neu tyrosine kinase in PC12 cells could be visualized by immunoprecipitation and Western blot analysis using a specific anti-Neu monoclonal antibody (Fig. 1*A*). Comparison of *lanes 1* and *2* (parental PC12 cells) and *lanes 5* and *6* (PC12 cells infected with the cDNA for the normal Neu tyrosine kinase; designated PC12/NeuN) in Fig. 1 indicates that although parental PC12 cells express Neu, a significantly stronger signal is obtained when Western blotting anti-Neu immunoprecipitates from PC12/NeuN cells. This signal is not affected by a 5-min incubation with heregulin in either the parental PC12 cells or the Neu transfectants.

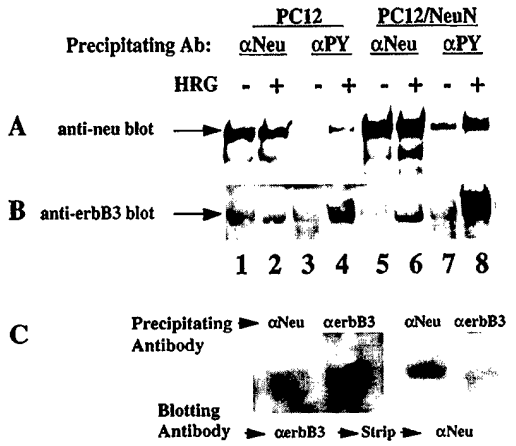

FIG. 1. **Heregulin-stimulated tyrosine phosphorylation of Neu/ErbB2 and ErbB3 and specificity of anti-ErbB3 antibody (*Ab*).** *A* and *B*, parental PC12 or PC12/NeuN (line I77.2, Ref. 9) cells (2×10^7 each) were incubated for 5 min with (+) or without (−) 10 nM heregulin (*HRG*) in serum-free medium, and cell lysates were prepared as described under "Materials and Methods." The lysates were divided equally for immunoprecipitation with antibodies against Neu (*αNeu*) or phosphotyrosine (*αPY*). *A*, the precipitates were analyzed by immunoblotting with antibody against Neu/ErbB2. *B*, a separate experiment in which the precipitates were analyzed by immunoblotting with the 2F12 monoclonal antibody against ErbB3. *C*, a lysate of PC12/NeuN cells was divided equally for immunoprecipitation with anti-Neu (*αNeu*) or anti-ErbB3 (*αErbB3*) antibodies. The precipitates were first analyzed by immunoblotting anti-ErbB3, and then the blot was stripped and reprobed with anti-Neu.

Lanes 3 and *4* in Fig. 1*A* also show that Neu is tyrosine-phosphorylated in a heregulin-stimulated manner in parental PC12 cells. As expected, the tyrosine phosphorylation of Neu is stronger in Neu transfectants (Fig. 1*A*, *lanes 7* and *8*), and in fact a doublet is detectable for Neu transfectants treated with heregulin.

Given that we previously had shown that the ErbB3 protein, by binding heregulin, enabled Neu to become responsive to heregulin (10), we examined whether the addition of heregulin to PC12 cells stimulated the formation of a complex between Neu/ErbB2 and ErbB3. We approached this in a separate experiment using a monoclonal antibody that is highly specific for ErbB3 and does not cross-react with the rat Neu protein (monoclonal 2F12, Ref. 15; see also Fig. 1*C*) as the first probe (Fig. 1*B*). In the parental PC12 cells, we had a difficult time detecting ErbB3 above background and have been unable to draw any conclusions regarding a heregulin-stimulated ErbB3-Neu complex. However, in cells that overexpress Neu, we can clearly detect ErbB3 in the anti-Neu precipitated pellets, and this appears to be a heregulin-stimulated event (see Fig. 1*B*, *lanes 5* and *6*). The fact that substantially more of this heregulin-stimulated ErbB3-Neu complex is found in the PC12/NeuN cells compared with the parental PC12 cells suggests that the affinity of Neu for ErbB3 is relatively weak, such that sufficient complex formation can only be detected by immunoprecipitation under conditions where the amount of one or the other of these proteins is relatively high (also see below).

The ErbB3 protein can be clearly detected in anti-phosphotyrosine immunoprecipitates (Fig. 1*B*, *lanes 4* and *8*). Again, the presence of ErbB3 in these pellets is entirely dependent upon the treatment of cells with heregulin, and the amount of ErbB3 is significantly increased in the PC12/NeuN cells compared with parental PC12 cells. Note that the broad ErbB3 band detected in anti-phosphotyrosine precipitates (Fig. 1*B*, *lane 8*) appears to contain a component with lesser electrophoretic mobility than that found in the anti-Neu precipitates

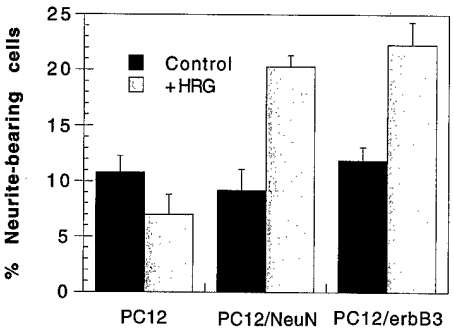

FIG. 2. **Heregulin-stimulated neurite outgrowth in PC12/ErbB3 (C59.3) cells.** Cells were incubated in 2 nM heregulin (*HRG*) in 0.5% fetal bovine serum for 3 days. Neurites were scored as described by Gamett and Cerione (9).

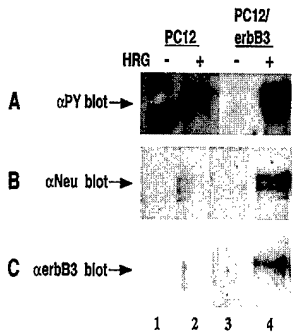

FIG. 3. **Heregulin stimulation of tyrosine phosphorylation of Neu and ErbB3 in parental PC12 and PC12/ErbB3 cells.** *A*, both immunoprecipitation and immunoblotting were done with anti-phosphotyrosine (*αPY*) antibodies. *B*, the cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the precipitates were analyzed by immunoblotting with anti-Neu antibody (*αNeu*). *C*, the cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the precipitates were analyzed by immunoblotting with the 2F12 monoclonal antibody against ErbB3 (*αErbB3*). *HRG*, heregulin.

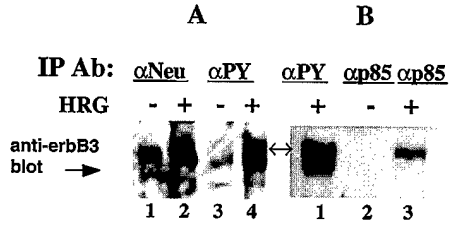

FIG. 4. **Anti-ErbB3 immunoblots showing association of different forms of ErbB3 protein with Neu and p85.** *A*, lysates from PC12/NeuN (A1.3) were immunoprecipitated with anti-Neu (*αNeu*) (*lanes 1* and *2*), and the supernatants after this precipitation were immunoprecipitated with anti-phosphotyrosine (*αPY*) (*lanes 3* and *4*). *B*, the lysate of heregulin-stimulated cells was divided equally for immunoprecipitation with antibodies against phosphotyrosine or p85 (*αp85*). The *arrow* points to the more slowly migrating form of ErbB3 discussed in the text. *IP Ab*, immunoprecipitation antibody; *HRG*, heregulin.

(this will be considered further below). It should also be noted that although the ErbB4 protein can be detected by Western blotting anti-phosphotyrosine precipitates with a specific anti-ErbB4 antibody, the apparent tyrosine phosphorylation of ErbB4 is not stimulated by heregulin in PC12 cells (data not shown).

Overexpression of ErbB3 in PC12 Cells—Overall, the data presented in Fig. 1 indicate that the ErbB3 protein interacts with Neu in a heregulin-dependent manner and that ErbB3 either is tyrosine-phosphorylated or associates with a tyrosine-phosphorylated protein in a heregulin- and Neu-dependent manner. One simple model consistent with these findings would be that heregulin stimulates the formation of a Neu-ErbB3 heterodimer, which in turn leads to the activation of the Neu tyrosine kinase and to the tyrosine phosphorylation of the ErbB3 protein. Presumably, the formation of this heterodimer would then initiate signaling pathways that account for the heregulin-stimulated neurite extension observed in PC12 cells (9). If this were in fact the case, we would predict that overexpression of ErbB3 would facilitate heregulin-stimulated neurite extension, as was observed upon overexpression of the normal Neu protein. In order to investigate this possibility, lipofection of the bovine *erbB3* cDNA was performed to generate stable PC12 cell transfectants that overexpress the ErbB3 protein. As shown in Fig. 2, PC12 cells overexpressing ErbB3 (PC12/ErbB3, clone C59.3) showed neurite extension that was stimulated by heregulin, similar to the response observed with PC12/NeuN cells.

Also similar to PC12/NeuN cells is the pattern of ~180-kDa proteins precipitated by the anti-phosphotyrosine antibody in a heregulin-dependent manner from PC12/ErbB3 cells (Fig. 3*A*). Western blotting with specific monoclonal antibodies indicates that these proteins include both Neu and ErbB3 (Fig. 3, *B* and *C*). In the case of Neu (Fig. 3*B*, *lane 4*), a doublet is detectable in the Western blot (also see Fig. 1*A*, *lane 8*), suggesting that there are multiple heregulin-stimulated phosphorylation sites. For ErbB3, we frequently detect a doublet in anti-phosphotyrosine immunoprecipitates of heregulin-stimulated cells, (*e.g.* Fig. 4); the more quickly migrating band was barely visible, however, in the experiment shown in Fig. 3*C*. The predominant ErbB3 band detected in anti-phosphotyrosine immunoprecipitates migrated more slowly than either band detected upon Western blotting these precipitates with the anti-Neu antibody. Taken together, these results suggest that both the Neu and ErbB3 proteins are tyrosine-phosphorylated in a heregulin-dependent manner in the ErbB3 transfectants and that a phosphorylated ErbB3 protein has distinctly lesser mobility than either of the phosphorylated forms of the Neu protein.

Interactions of Distinct Forms of ErbB3 with Neu and the 85-kDa Regulatory Subunit (p85) of the Phosphatidylinositol 3-Kinase in PC12 Cells—Based on considerations of consensus sequences (16, 17), it has been predicted that the phosphorylated ErbB3 protein would bind to the 85-kDa regulatory subunit of the phosphatidylinositol 3-kinase. Indeed, binding of the ErbB3 cytoplasmic domain to p85 has been demonstrated following EGF stimulation of fibroblasts expressing an EGF receptor-ErbB3 chimera (18, 19) and ErbB3 has been shown to interact with p85 following EGF stimulation of MDA-MB-468 breast cancer cells (15) and A431 cells (20). Having seen heregulin-dependent tyrosine phosphorylation of ErbB3 in PC12 cells, we wanted to examine whether this would also lead to the interaction of ErbB3 and p85. Furthermore, the previous results (Figs. 1 and 3) had shown the existence of two or more tyrosine-phosphorylated species of ErbB3 in heregulin-treated cells, whereas only a single ErbB3 band appeared to associate with Neu, thus raising the possibility of distinct roles for the different forms of ErbB3. These questions were addressed by the experiments shown in Fig. 4. *Lanes 1* and *2* in Fig. 4*A* show the heregulin-dependent co-precipitation of ErbB3 in anti-Neu immunoprecipitates from lysates of PC12/NeuN cells. The supernatants remaining after anti-Neu precipitation were found to contain an additional, slower mobility ErbB3 band (see Fig. 4*A*, *arrow*, *lane 4*) precipitable by anti-phosphotyrosine. The appearance of this band is absolutely dependent on the addition of heregulin. These results suggest that although Neu and ErbB3 can be co-precipitated from PC12 cells overexpressing Neu, and while ErbB3 may be tyrosine-phosphorylated within this complex, there is a second phosphorylated form of

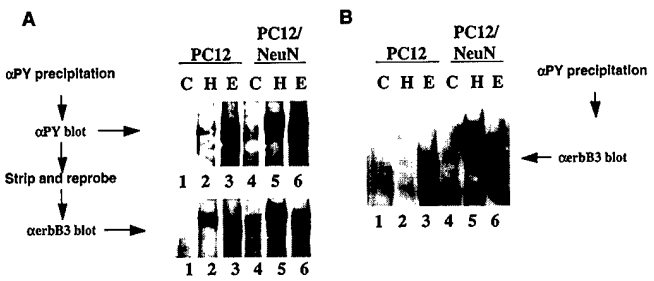

FIG. 5. **Comparison of heregulin- and EGF-stimulated tyrosine phosphorylation of ErbB3.** *A*, PC12 or PC12/NeuN (I77.2) cells were stimulated for 5 min with 10 nM heregulin or 100 ng/ml EGF (lanes marked *H* and *E*, respectively; untreated controls are marked *C*). Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (*αPY*), and the precipitates were analyzed by immunoblotting with anti-phosphotyrosine antibodies (*top*). Then the blot was reprobed with anti-ErbB3 (*αErbB3*, *bottom*). *B*, PC12 or PC12/NeuN (I77.2) cells were treated as described in *A*, then cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the precipitates were analyzed by immunoblotting with anti-ErbB3 antibody.

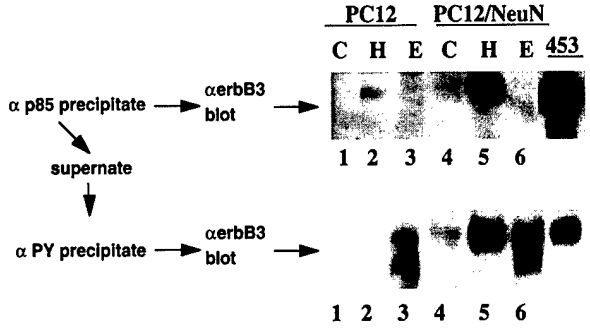

FIG. 6. **Comparison of heregulin- and EGF-stimulated complex formation between ErbB3 and p85.** PC12 and PC12/NeuN (I77.2) cells were stimulated with heregulin or EGF as described in the legend for Fig. 5. Cell lysates were immunoprecipitated with anti-p85 antibodies (*αp85*), and the supernatants were then immunoprecipitated with anti-phosphotyrosine antibodies (*αPY*). Both the anti-p85 (*top*) and anti-phosphotyrosine (*bottom*) precipitates were analyzed by immunoblotting with ErbB3 antibodies (*αErbB3*).

ErbB3 that migrates with slower mobility and is not complexed with Neu (although the generation of this ErbB3 species is dependent on heregulin and is enhanced by overexpression of Neu).

Interestingly, we find that it is this ErbB3 species that can be co-precipitated with an anti-p85 antibody (Fig. 4*B*). Specifically, *lane 1* in Fig. 4*B* shows the ErbB3 doublet obtained when Western blotting an anti-phosphotyrosine precipitate from PC12 cells overexpressing Neu, and *lanes 2* and *3* show a similar experiment using a specific anti-p85 antibody to precipitate ErbB3. In direct comparisons, we consistently find that the form of ErbB3 that complexes with p85 has an identical mobility to the upper ErbB3 band detected in anti-phosphotyrosine immunoprecipitates. Again, the ability to co-precipitate ErbB3 and p85 is strictly dependent on heregulin addition to the PC12 cells. These results are consistent with a model where heregulin stimulates the formation of a Neu-ErbB3 complex and the multiple phosphorylation of ErbB3 (see "Discussion"). One phosphorylated form of ErbB3 is able to remain complexed with Neu, whereas a second phosphorylated form, which migrates with a slower mobility on SDS-polyacrylamide gel electrophoresis, dissociates from Neu and is able to form a complex with p85.

Comparisons of the Effects of Heregulin and EGF on the Tyrosine Phosphorylation of ErbB3 and the Interactions of ErbB3 with p85—Given that in other cell systems it has been shown that the EGF receptor can couple functionally to ErbB3 and p85 (15, 20), we compared the abilities of EGF and heregulin to stimulate the phosphorylation of ErbB3 and its complex formation with p85. The *upper panel* in Fig. 5*A* compares the results from Western blot analyses (using an anti-phosphotyrosine antibody) where tyrosine-phosphorylated proteins were immunoprecipitated from parental PC12 cells and PC12/NeuN cells, in both the presence and the absence of treatment with heregulin or EGF. The predominant bands observed in these experiments are at ~180 kDa, which is the expected mobility for the EGF receptor and the related Neu and ErbB3 proteins. The EGF-stimulated phosphorylation signals in the parental PC12 cells and in PC12/NeuN cells appeared to be equivalent (compare *lanes 3* and *6* in the *upper panel* of Fig. 5*A*), whereas, as indicated earlier (*e.g.* Fig. 1), the heregulin response in PC12/NeuN cells was enhanced relative to the parental PC12 cells (compare *lanes 2* and *5* in the *upper panel* of Fig. 5*A*). The results obtained in PC12 cells overexpressing Neu also clearly show that the mobilities of the phosphoprotein bands in the ~180-kDa region differ for heregulin-treated cells compared with EGF-treated cells. Heregulin treatment results in phosphoprotein bands that have less mobility than those detected following EGF-stimulation (compare *lanes 5* and *6* in the *upper panel* of Fig. 5*A*). These results suggest either that different proteins are being tyrosine-phosphorylated upon the addition of EGF *versus* heregulin and/or that different sites are being phosphorylated on the same proteins.

When the same anti-phosphotyrosine precipitates are reblotted with an anti-ErbB3 antibody, the overall profile obtained is very similar to that observed in the anti-phosphotyrosine blot (Fig. 5*A*, *lower panel*). This similarity argues that both heregulin and EGF stimulate the tyrosine phosphorylation of ErbB3 and that the stimulatory effects of heregulin but not EGF are greatly enhanced in PC12 cells overexpressing Neu. In order to be certain that these results were not artifactual due to incomplete stripping of anti-phosphotyrosine antibody prior to reprobing, we repeated the experiment and blotted first with anti-ErbB3 (Fig. 5*B*). Again, the electrophoretic mobilities of the ErbB3 bands are clearly different for heregulin and EGF treatment (compare *lanes 5* and *6* of Fig. 5*B*). The heregulin-stimulated cells showed the two forms of ErbB3 described above (Fig. 4). The faster mobility ErbB3 band observed upon heregulin treatment co-migrated with the slower mobility EGF-stimulated ErbB3 band. These results suggest that both the EGF receptor and Neu can elicit a common phosphorylation event within the ErbB3 protein and that this phosphorylated ErbB3 species (when generated by heregulin treatment) can remain complexed with Neu. There also is a broad ErbB3 band detected in EGF-treated cells that has a greater mobility than any of the bands detected in heregulin-treated cells.

The experiment shown in Fig. 6 tested for the formation of stable complexes between the various phosphorylated forms of ErbB3 seen in heregulin- and EGF-stimulated cells with p85. For this, lysates of parental PC12 cells or PC12/NeuN cells were first immunoprecipitated with an anti-p85 antibody, and the precipitates were blotted with the anti-ErbB3 antibody (Fig. 6, *upper panel*). The supernatants remaining after the anti-p85 precipitation were then immunoprecipited with anti-phosphotyrosine antibodies, and these precipitates were also analyzed by blotting with anti-ErbB3 (Fig. 6, *lower panel*). As shown in the *upper panel*, we only detect a p85-ErbB3 complex in heregulin-treated cells (Fig. 6, *lanes 2* and *5*) and not in EGF-treated cells (Fig. 6, *lanes 3* and *6*), and this complex is strongly stimulated in PC12 cells overexpressing Neu (compare *lanes 2* and *5* of Fig. 6). Again, the mobility of the ErbB3 band that co-precipitates with p85 is identical to the slower mobility ErbB3 band detected in anti-phosphotyrosine immunoprecipi-

tates. When examining the lysates that remain behind following the immunoprecipitation with anti-p85, we see both heregulin- and EGF-stimulated phosphoprotein bands in the ~180-kDa region. Thus, whereas EGF stimulates the phosphorylation of ErbB3 (as indicated in Fig. 5), this phosphorylated ErbB3 species is not able to form a stable complex with p85. As will be discussed further below, these results then point to a model where both the EGF-activated EGF receptor and the heregulin-activated Neu tyrosine kinase can phosphorylate ErbB3 in a distinct manner and that only ErbB3 proteins that have been phosphorylated by Neu can go on to form a stable complex with p85 in PC12 cells.

DISCUSSION

It originally was reported that heregulin, a 144-kDa transmembranal glycoprotein with an EGF-like region within its extracellular domain, was the ligand/growth factor for Neu/ErbB2 (5). However, recent studies have indicated that the true receptors for heregulin are the ErbB3 and ErbB4 proteins (10, 12) and that it is heterodimer formation between Neu and ErbB3 (or Neu and ErbB4) that enables the Neu tyrosine kinase activity to be stimulated by heregulin (11). Here we looked for the possible involvement of ErbB3 or ErbB4 in the neurite outgrowth and tyrosine phosphorylation responses we had previously seen associated with overexpression of Neu in PC12 cells. We found that heregulin stimulates the tyrosine phosphorylation of endogenous ErbB3 protein in PC12 cells and that this phosphorylation, like that of Neu, is greatly enhanced in cells that overexpress Neu. Furthermore, we found that overexpressing the ErbB3 protein in PC12 cells led to heregulin-stimulated neurite extension, similar to the phenotypes obtained upon heregulin addition to cells overexpressing the normal Neu protein or when transforming Neu was expressed in PC12 cells. Two obviously important questions were whether the Neu and ErbB3 proteins actually formed a complex in PC12 cells and if this complex formation was stimulated by heregulin. The results of immunoprecipitation experiments using anti-Neu antibody indicated that the addition of heregulin either to PC12 cells overexpressing Neu or to PC12 cells overexpressing ErbB3 led to the co-precipitation of the Neu and ErbB3 proteins. Thus, overall, these results are consistent with a scheme where heregulin-stimulated heterodimer formation between Neu and ErbB3 results in the increased tyrosine phosphorylation of the Neu and ErbB3 proteins (see below) and accounts for the ability of PC12 cells to respond to this growth factor.

Because it has been suggested that one of the primary effector/targets for phosphorylated ErbB3 is the 85-kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (18, 19, 20), we set out to determine if heregulin addition to PC12 cells might lead to the formation of an ErbB3-p85 complex. Immunoprecipitation experiments using a specific (precipitating) anti-p85 antibody followed by Western blotting with a specific anti-ErbB3 antibody provided evidence for a direct interaction between these proteins. This interaction was most evident in PC12 cells overexpressing Neu and was heregulin-stimulated. When comparing anti-phosphotyrosine immunoprecipitates with anti-85 immunoprecipitates, where in both cases the resuspended pellets were blotted with the anti-ErbB3 antibody, we found that the ErbB3 band that co-precipitated with p85 was identical in mobility to the slowest mobility ErbB3 band detected in anti-phosphotyrosine precipitates. However, this ErbB3 band was not detected in anti-Neu immunoprecipitates. Thus, although it appears that ErbB3 is phosphorylated at multiple tyrosine residues in a heregulin- and Neu-dependent manner, one of these phosphorylated ErbB3 species forms a stable complex with Neu but not with p85.

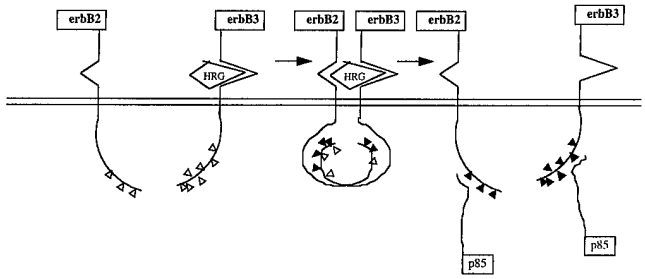

FIG. 7. **A model for heregulin signal transduction involving Neu/ErbB2 and ErbB3 in PC12 cells.** Binding of heregulin (*HRG*) to ErbB3 is followed by formation of a heterodimer of Neu/ErbB2 and ErbB3, within which the Neu tyrosine kinase is activated and ErbB3 becomes phosphorylated. Phosphorylated ErbB3 and Neu/ErbB2 independently complex with p85.

Overall, these results appear to be consistent with the simple scheme depicted in Fig. 7. The binding of heregulin to ErbB3 on the surface of PC12 cells stimulates the formation of a Neu-ErbB3 heterodimer. We would suggest that this results in a higher affinity binding by heregulin (11) and the stimulation of the Neu tyrosine kinase activity. This in turn increases the tyrosine phosphorylation of Neu, probably at multiple sites (*e.g.* Fig. 1), and the trans-phosphorylation of multiple tyrosine residues on ErbB3. The specific mechanism by which heregulin stimulates the phosphorylation of Neu is not clear. One possibility is that Neu is trans-phosphorylated by ErbB3; however, this is problematic because ErbB3 appears to have little or no tyrosine kinase activity (14). It may be that within a Neu-ErbB3 heterodimer, a weak kinase like ErbB3 is still able to phosphorylate Neu because of the immediate proximity of the substrate. Once activated, the Neu tyrosine kinase should be able to trans-phosphorylate ErbB3. The timing of this trans-phosphorylation event apparently is important, because the extent of ErbB3 phosphorylation appears to influence whether or not ErbB3 remains complexed with Neu or forms a new complex with p85. In fact, these findings raise the interesting possibility that a specific, heregulin-stimulated trans-phosphorylation of ErbB3 by Neu leads to the dissociation of ErbB3 from the heregulin-Neu-ErbB3 ternary complex and promotes the specific binding of ErbB3 to a potential target, p85. Such a mechanism would be similar to hormone receptor/G protein-mediated signaling cascades where the G protein first becomes activated within a hormone-receptor-G protein ternary complex, but then the activated G protein dissociates from this complex and seeks out its target/effector molecule.

It also is interesting that a potential signaling cascade involving ErbB3 and p85 within PC12 cells appears to be specifically initiated by heregulin and not by EGF. Although the addition of EGF to PC12 cells overexpressing Neu leads both to an apparent tyrosine phosphorylation of Neu (data not shown) and ErbB3 (Fig. 5*A*), neither of these phosphorylated proteins appear to form a stable complex with p85. In the future, it will be interesting to see if the heregulin-Neu-ErbB3-p85 pathway contributes to the specificity observed in the cellular responses triggered by heregulin *versus* EGF.

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Secondary Dimerization between Members of the Epidermal Growth Factor Receptor Family*

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Growth factor receptors of the epidermal growth factor (EGF) receptor family play pivotal roles in the regulation of cell proliferation and differentiation and are involved in the development of human cancers. It has been well documented that these receptors undergo growth factor-stimulated homo- and heterodimerization as a first step in the initiation of signaling cascades. Here we provide evidence for a new mechanism for growth factor-stimulated receptor dimer formation, designated secondary dimerization. The growth factor-induced dimerization and ensuing receptor trans-autophosphorylation results in the dissociation of the original (primary) receptor dimer. Each phosphorylated receptor monomer then interacts with a new (nonphosphorylated) receptor to form a secondary dimer. Treatment of cells with EGF yields Neu-ErbB3 secondary dimers, and heregulin treatment induces the formation of Neu-EGF receptor (secondary) dimers. The ability of EGF and heregulin to stimulate a cascade of dimerization events points to a novel mechanism by which multiple signaling activities and diverse biological responses are initiated by members of the EGF receptor family.

The interactions of various growth factors and cytokines with cell surface receptor tyrosine kinases initiate a variety of intracellular signaling pathways that when integrated yield cell cycle progression, cell differentiation, or apoptosis. Several classes of receptor tyrosine kinases have been described, among which the EGFR family is of particular interest, because these receptors have been implicated in malignant transformation (1–8). This family includes the epidermal growth factor (EGF)[1] receptor (also referred to as ErbB1), the Neu/ErbB2 protein (referred to from here on as Neu), and the more recently identified ErbB3 and ErbB4 proteins (9–12). Two types of ligands interact with members of the EGFR family; EGF is a prototype for ligands that bind to the EGFR, and heregulin (HRG) or Neu differentiation factor represents a family of ligands that bind to both ErbB3 and ErbB4 (10, 13–15). Two of the receptors, EGFR and ErbB4, are capable of ligand-stimulated tyrosine kinase activity. Thus far, the Neu tyrosine kinase is an orphan receptor, whereas ErbB3 appears to be a kinase-defective receptor (14, 16).

The binding of EGF or HRG to their receptors results in receptor dimerization and receptor trans-autophosphorylation. The phosphorylated receptors recruit cellular signaling proteins, through the binding of their Src homology 2 or phosphotyrosine binding domains and thus initiate signaling pathways (17, 18). The binding of EGF stimulates the formation of both EGFR homodimers or heterodimers like EGFR-Neu (19–21). Similarly, HRG can stimulate receptor homodimer formation of ErbB3 or of ErbB4 as well as receptor heterodimers like ErbB3 and Neu or ErbB4 and Neu (13, 15). The HRG-promoted formation of these heterodimers provides the molecular basis for the stimulation of Neu tyrosine kinase activity (7, 13, 15, 22).

The mode of heterodimerization between members of the EGFR family may have a significant influence on malignant transformation. For example, the co-expression of ErbB3 and Neu in NIH3T3 cells results in neoplastic transformation, whereas neither the expression of ErbB3 nor Neu alone is sufficient for transformation (1, 7). The heterodimerization of the EGFR with Neu results in cell transformation, whereas the replacement of wild type Neu by its kinase-defective counterpart abrogates transformation (2, 23). Moreover, the expression of Neu in breast and ovarian carcinomas correlates with a poor prognosis (1, 2, 5, 6). The molecular basis for this is likely due to an enhanced tendency of the orphan Neu receptor to form heterodimers with other members of the EGFR family (16, 22, 24).

In this study we present evidence for a new mode of inter-receptor interaction, designated secondary dimerization. Our results show that ligand-induced dimer formation might be followed by dimer dissociation and interaction of the individual, activated receptors with inactive receptors to form new, secondary dimers.

MATERIALS AND METHODS

Cells, Growth Factors, Antibodies, and Inhibitors—Rat pheochromocytoma PC12 cell line I77.2 (25, 26) was used in this study. Recombinant heregulin (rHRG-$\beta1_{177-241}$) was provided by Dr. Mark Sliwkowski (Genetech, Inc.), and EGF was purchased from Sigma. Anti-EGFR polyclonal antibodies (SC 03) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-c-Neu monoclonal antibodies (Ab-4) were from Calbiochem (San Diego, CA), anti-ErbB3 (2F12) and anti-c-ErbB4 (AB-2) monoclonal antibodies were from Neo Markers (Fremont, CA), and anti-phosphotyrosine monoclonal antibodies (PY20) were from Transduction Laboratories (Lexington, KY). Antibodies were tested for their specificity, and no cross-reactivity was detected. Tyrphostins were purchased from Calbiochem (San Diego, CA).

Cell Growth, Immunoprecipitation, Western Blots, and Determination of Tyrosine Kinase Activity—PC12 cells were grown on 15-cm plastic dishes in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 units/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Amphotericin B (Sigma) in a CO_2 incubator (5% CO_2) at 37 °C.

Cell stimulation, immunoprecipitation, and Western blotting were carried out as described previously (26). Nearly confluent cell cultures were incubated for 16–20 h in growth medium containing 0.1% serum. Then the cells were gently detached by a short incubation with Hanks'

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin.

balanced salt solution (without calcium or magnesium), centrifuged at 500 × *g* for 5 min, and resuspended in serum-free growth medium. The use of cell suspensions enables the stimulation of a large number of cells in each sample. In control experiments, we did not find a difference between the results obtained when using either attached cells or suspended cells.[2] Cells were dispensed at 1.0×10^7 cells/1.5 ml in Eppendorf tubes, and HRG or EGF (20 nM or 100 ng/ml, respectively) was added to some of the tubes for 2 min at 37 °C. Cells subjected to treatment with tyrosine kinase inhibitors were preincubated for 2 min at 37 °C with tyrphostins before the addition of the growth factors. Tyrphostins AG556, AG879, and AG1478 were added to final concentrations of 25, 25, and 0.5 μM, respectively. The incubations were terminated by transferring the tubes to ice, followed by immediate centrifugation to pellet the cells and removal of the medium. The cell pellets were treated with cold lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidat P-40, 10% glycerol, 1 mM phenylmethysulfonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 mM EGTA, 40 mM sodium fluoride, 0.1 mM ammonium molybdate, and 1 mM sodium orthovanadate. After 15 min of incubation on ice, the cell lysates were centrifuged for 10 min at 15,000 × *g* to remove insoluble material. Antibodies were then added to the lysates, as needed for each experiment, followed by 30 μl of a 50% suspension of protein A-Sepharose (Sigma). The tubes were incubated for 3 h in the cold with slow agitation, and then the immunocomplexes were pelleted by centrifugation, washed once with lysis buffer, and boiled for 3 min in 40 μl of Laemmli sample buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis (8% acrylamide), and electrophoretically transferred to polyvinilidene difluoride membranes (Immobilon-P, Millipore, Burlington, MA). The blots were blocked by 1 h of incubation with 3% bovine serum albumin in Tris-buffered saline with 0.05% Tween 20, washed, and incubated for 1 h with primary antibodies as indicated. Blots were analyzed by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents (Amersham Corp.).

Determination of tyrosine kinase activity in the immunoprecipitates was performed as described previously (27). Cell growth, cell exposure to either HRG or EGF, cell lysis, and immunoprecipitation were carried out as described above for immunoblotting. Each immunoprecipitate was washed twice with a buffer containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 mM sodium vanadate and resuspended in 60 μl of the same buffer. The assay was initiated by the addition of 20 μl of immunoprecipitate to 20 μl of reaction mixture containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 mM sodium vanadate, 10 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 0.5 mg/ml poly-Glu_4-Tyr_1. Some of the reaction mixtures contained tyrphostins, either AG879, AG556, or AG1478, at the respective concentrations of 50, 50, and 1 μM. The assays were conducted for 10 min at room temperature and were terminated by the addition of 10 μl of 0.5 M EDTA. The tubes were centrifuged, and the supernatants were transferred to Whatman 3MM paper strips, which were incubated overnight in 10% trichloroacetic acid, containing 10 mM sodium pyrophosphate at 4 °C, with gentle agitation. The paper strips were then dried and counted in a scintillation counter.

Assays of tyrosine kinase activity of receptors expressed in insect cells were carried out as described previously (28, 29). Briefly, membranes prepared from insect cells (Sf21) preinfected with recombinant viruses (28) harboring either *erb1*, *neu*, or *erbB4* (the latter was obtained from K. L. Carraway (Department of Cell Biology Harvard Medical School, Boston, MA)) were incubated for 10 min at room temperature in a 40-μl reaction mixture containing 20 mM HEPES buffer, pH 7.4, 5 mM $MnCl_2$, 0.5 mM sodium vanadate, 0.5 mg/ml poly-Glu_4-Tyr_1, and 5 μM of [γ-^{32}P]ATP (10 Ci/mmol). The reaction was terminated by the addition of 10 μl of 5 × Laemmli sample buffer (30), and samples were subjected to SDS-polyacrylamide gel electrophoresis (8% acrylamide). Bands of interest were excised from the dried gels, and the associated radioactivity was counted in a scintillation counter (28, 29). Receptors were expressed in insect cells infected with viruses harboring the receptor genes but not in noninfected cells or in cells infected with the wild type virus, as was determined by Western blots.

RESULTS AND DISCUSSION

Indications for a new mode of inter-receptor interactions between members of the EGFR family were obtained during a study of the effects of tyrphostins, specific inhibitors of tyrosine kinase activity (31), on receptor dimerization and tyrosine kinase activity in PC12 cells. In these experiments, cells were exposed to EGF or HRG and then subjected to lysis and immunoprecipitation by anti-EGFR or anti-Neu antibodies. The tyrosine kinase activity in the immunoprecipitates was inhibited by the tyrphostin AG556, a specific inhibitor of EGFR, and by AG879, which specifically inhibits Neu in various cells (27, 31–33), including PC12 cells (34).

TABLE I

Effect of tyrphostins on tyrosine kinase activity

PC12 cells were treated with a ligand and lysed, and receptors were immunoprecipitated. Tyrosine kinase activity was determined in the immunoprecipitates as described under "Materials and Methods" and in Ref. 27.

Ligand	IP[a]	Tyrphostin	TK[b]
EGF	Anti-EGFR	AG879	0.50 ± 0.22
		AG556	0.39 ± 0.15
EGF	Anti-Neu	AG879	0.56 ± 0.15
		AG556	0.53 ± 0.19
HRG	Anti-Neu	AG879	0.52 ± 0.16
		AG556	0.30 ± 0.02
HRG	Anti-EGFR	AG879	0.89 ± 0.04
		AG556	0.57 ± 0.23

[a] Immunoprecipitation.

[b] Relative activity of tyrosine kinase, as compared with untreated cells.

The results of these studies suggested that both the EGFR and Neu tyrosine kinase activities were present in anti-EGFR immunoprecipitates (Table I) and were consistent with previous findings in other cell types (16, 21), as well as studies in PC12 cells,[2] which indicated that EGF treatment induced the formation of EGFR-Neu heterodimers. This was further reinforced by the data shown in Table I, where the tyrosine kinase activity measured in anti-Neu immunoprecipitates was sensitive to both the EGFR and Neu antagonists. However, unlike the results obtained with EGFR and Neu immunoprecipitates from EGF-stimulated PC12 cells, the data obtained from HRG-stimulated cells were unexpected. In particular, immunoprecipitation of Neu from HRG-treated PC12 cells yielded tyrosine kinase activity that was not only sensitive to the Neu kinase inhibitor (AG879) but also to the EGFR kinase inhibitor (AG556) (Table I). This finding indicates the possibility that EGFR is present in anti-Neu immunoprecipitate, although HRG does not serve as a ligand for EGFR. Likewise, anti-EGFR immunoprecipitates from HRG-treated PC12 cells showed some sensitivity to the Neu kinase inhibitor (AG879) in addition to the EGFR kinase inhibitor (AG556) (Table I), indicating the presence of Neu in the immunoprecipitate.

Using insect cell expression systems for members of the EGFR family (EGFR, Neu, or ErbB4), we have verified that AG879 shows a strong preference for Neu *versus* EGFR and is incapable of inhibiting ErbB4 activity (Table II). However, both AG556 and AG1478, which were reported to be specific for the EGFR (31, 33), are also capable of inhibiting ErbB4 tyrosine kinase activity (Table II).

Thus, one possible explanation for the results obtained with the anti-Neu immunoprecipitates (*e.g.* Table I) was that HRG stimulated the formation of an ErbB4-Neu primary heterodimer, in which the tyrosine kinase activity of ErbB4 would be sensitive to AG556. Thus far, however, we have not been able to reliably detect the formation of a HRG-stimulated ErbB4-Neu heterodimer in PC12 cells through Western blot analysis, using specific anti-ErbB4 antibodies. An additional possibility, which would not be mutually exclusive with the first, was that HRG stimulated the formation of a Neu-EGFR heterodimer complex. In fact, as shown in Fig. 1 (also, see Fig. 2, below), anti-Neu immunoprecipitates from HRG-treated PC12 cells contain EGFR, as well as Neu and ErbB3.

[2] D. Gamett, unpublished data.

TABLE II

Effect of tyrphostins on tyrosine kinase activity of receptors expressed in insect cells

The values in the table represent relative tyrosine kinase activities, as compared with control systems without tyrphostin. Procedures for the determination of the enzymatic activities are described under "Materials and Methods" and in Refs. 28 and 29.

Tyrphostin	EGFR		Neu		ErbB4	
	S[a]	R[b]	S	R	S	R
AG556	0.23	0.11	1.21	1.03	0.32	0.35
AG1478	0.09	0.09	0.88	1.06	0.29	0.19
AG879	0.87	0.74	0.20	0.38	1.16	0.81

[a] Phosphorylation of a substrate (poly-Glu_4-Tyr_1).
[b] Receptor autophosphorylation.

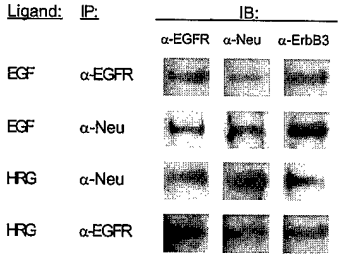

FIG. 1. **Receptors present in immunoprecipitates.** PC12 cells in culture were starved for 1 day in serum-free medium, detached, and suspended in serum-free medium. Either HRG (20 nM) or EGF (100 ng/ml) was added to the cells for 2 min, after which the cells were lysed, and the cell lysates were subjected to immunoprecipitation (*IP*) using either anti-EGFR or anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-EGFR, anti-Neu, or anti-ErbB3 antibodies followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. Bands were not detected after immunoblotting with anti-ErbB4 antibodies.

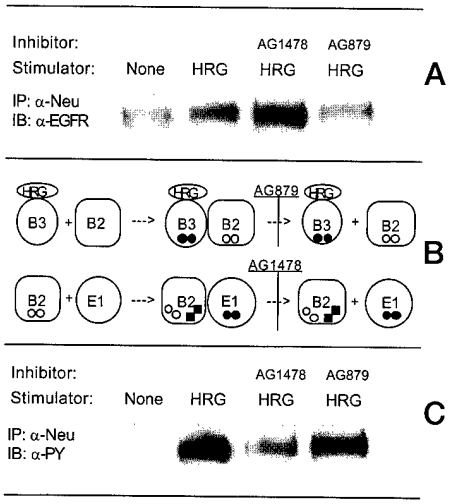

FIG. 2. **HRG induces the association of Neu with EGFR.** *A*, PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. Some cell samples were preincubated for 2 min with tyrphostin AG1478 (0.5 μM) or AG879 (25 μM). Then the cells were stimulated for 2 min with HRG (20 nM) and lysed, and the cell lysates were subjected to immunoprecipitation (*IP*) using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-EGFR antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. *B*, cell stimulation by HRG results in the formation of the primary heterodimer ErbB3-Neu, followed by trans-phosphorylation, dimer dissociation, and interaction of the phospho-activated Neu with EGFR to form the secondary heterodimer Neu-EGFR. The tyrphostin AG879 inhibits the tyrosine kinase activity of Neu and thus arrests the dissociation of the primary dimer Neu-ErbB3 and the formation of the secondary dimer Neu-EGFR. The tyrphostin AG1478 inhibits EGFR tyrosine kinase activity and thus arrests the dissociation of the secondary dimer Neu-EGFR. *E1*, EGFR; *B2*, Neu; *B3*, ErbB3. *Light gray* and *dark gray symbols* represent low and high phosphorylation levels, respectively. *C*, the immunoblot (*IB*) described in *A* was subjected to a second blotting with anti-phosphotyrosine antibodies.

Because neither the EGFR nor Neu directly binds HRG, these findings suggest that the EGFR-Neu complex is a secondary outcome of a primary heterodimerization event stimulated by HRG. We suggest that the trans-phosphorylation in the HRG-stimulated ErbB3-Neu dimer might be followed by dimer dissociation and interaction between activated Neu and a latent EGFR. This suggestion is in concert with our previous study, in which we have shown that HRG stimulates the heterodimerization between ErbB3 and Neu in PC12 cells but that following the trans-phosphorylation of ErbB3, it dissociated from Neu and formed a complex with the 85-kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (26). Taken together, these results led us to hypothesize that the HRG-stimulated ErbB3-Neu (primary) heterodimer was not stable under conditions of trans-phosphorylation, such that the phosphorylation of the individual receptor (within the primary heterodimer) causes dimer dissociation, thus making the resulting monomers available to interact with other signaling partners. In some cases, the phosphorylated monomeric receptors may bind to Src homology 2 domain-containing proteins like p85. In other cases, however, the phosphorylated monomers can apparently interact with other (nonphosphorylated) receptor monomers to form secondary dimers.

If this hypothesis were correct, we would predict that treatment with different tyrphostins, specific inhibitors of tyrosine kinase activity (31), should have specific effects on the formation of secondary receptor dimers. The data presented in Fig. 2*A* (*lanes 3* and *4*) show that this in fact was the case. The treatment of PC12 cells with AG1478, which is a potent and specific antagonist for EGFR in various cells (31, 33, also see Table II), increased the formation of the HRG-stimulated Neu-EGFR secondary dimer, whereas, treatment with AG879, the Neu tyrosine kinase antagonist (31, 33, 34), strongly inhibited the formation of the Neu-EGFR secondary dimer. These findings are fully consistent with the model shown in Fig. 2*B*. Namely, the tyrphostin AG879, by inhibiting Neu tyrosine kinase activity, prevents both the autophosphorylation of Neu and the trans-phosphorylation of ErbB3 and thus prevents the dissociation of the primary Neu-ErbB3 dimer. This, then, inhibits the formation of secondary Neu-EGFR heterodimers. However, tyrphostin AG1478, by inhibiting the EGFR tyrosine kinase activity, prevents the trans-phosphorylation of Neu within the secondary Neu-EGFR dimer, hence inhibiting the dissociation of this dimer, and increases its amount.

Fig. 2*C* shows the results of a Western blot of Neu immunoprecipitates using an anti-phosphotyrosine antibody. *Lanes 1* and *2* show that HRG treatment of PC12 cells stimulated the tyrosine phosphorylation of the receptors present in the anti-Neu immunoprecipitate. This phosphorylation was strongly inhibited by AG1478 (Fig. 2*C*, *lane 3*), which argues that the majority of the phosphorylation occurred (in an EGFR-catalyzed manner) within the secondary Neu-EGFR dimer. The tyrosine phosphorylation of Neu was only weakly inhibited by

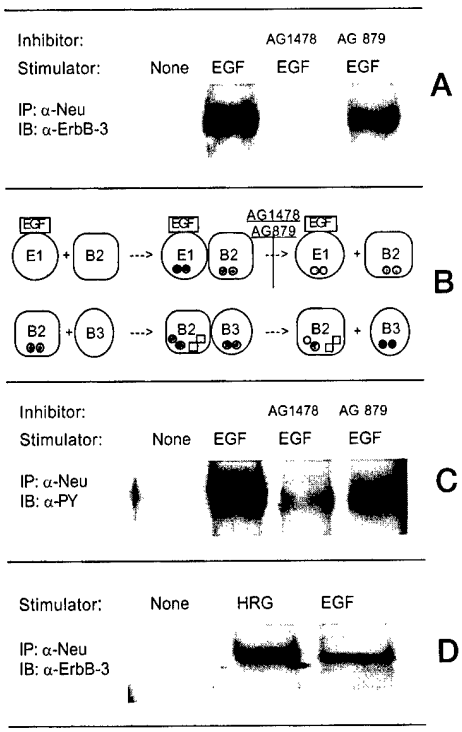

FIG. 3. **EGF induces the association of Neu with ErbB3.** *A*, PC12 cells in culture were starved for 1 day in serum-free growth medium, detached, and suspended in serum-free medium. The cells were stimulated for 2 min with EGF (100 ng/ml) and then lysed, and the cell lysates were subjected to immunoprecipitation (*IP*) using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence. *B*, EGF induces the formation of the heterodimer Neu-EGFR. Trans-phosphorylation leads to dimer dissociation followed by the interaction of activated Neu with ErbB3 to form the secondary dimer Neu-ErbB3. The tyrphostins AG1478 and AG879 inhibit the trans-phosphorylation and the dissociation of the primary dimer EGFR-Neu and thus decrease the levels of the secondary dimer Neu-ErbB3. *E1*, EGFR; *B2*, Neu; *B3*, ErbB3. *Light gray* and *dark gray symbols* represent low and high phosphorylation levels, respectively. *C*, the immunoblot (*IB*) described in *A* was subjected to a second blotting with anti-phosphotyrosine antibodies. *D*, cells were stimulated for 2 min with either HRG or EGF (20 nM or 100 ng/ml, respectively). Then the cells were lysed, and the cell lysates were subjected to immunoprecipitation using anti-Neu antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-ErbB3 antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. The bands were detected by chemiluminescence.

AG879 (Fig. 2*C*, *lane 4*), which suggests that Neu autophosphorylation represented only a small percentage of the total tyrosine phosphorylation of Neu.

The data shown in Fig. 3 provide evidence for the formation of another type of secondary dimer. In this case, the stimulation of PC12 cells with EGF led to the formation of a dimer between Neu and ErbB3. Again, because neither of these receptors bind EGF, this dimerization event must be the secondary outcome of a primary EGF-stimulated dimerization between the EGFR and Neu. The formation of this secondary receptor dimer was strongly inhibited by the EGFR antagonist, AG1478, and moderately inhibited by the Neu antagonist, AG879 (Fig. 3*A*). These results are consistent with the model depicted in Fig. 3*B*. The marked inhibition of (secondary) Neu-ErbB3 dimer formation by AG1478 suggests that the EGFR-catalyzed trans-phosphorylation of Neu is essential for the dissociation of Neu from the EGFR (within the primary dimer). The modest inhibition of (secondary) Neu-ErbB3 dimer formation by AG879 argues that Neu autophosphorylation and EGFR trans-phosphorylation (by Neu) are less critical for the

TABLE III
Selection of primary and secondary dimers by specific ligands and selective immunoprecipitation

Underlined receptors are present only in secondary dimers under the selective conditions used.

Ligand	IP[a]	Primary dimers	Secondary dimers
HRG	Anti-Neu	Neu-ErbB3	EGFR-Neu
		Neu-ErbB4	Neu-Neu
HRG	Anti-EGFR	EGFR-ErbB3	EGFR-EGFR
		EGFR-ErbB4	EGFR-Neu
EGF	Anti-Neu	EGFR-Neu	Neu-Neu
			Neu-ErbB3
			Neu-ErbB4
EGF	Anti-EGFR	EGFR-EGFR	
		EGFR-Neu	
		EGFR-ErbB3	
		EGFR-ErbB4	

[a] Immunoprecipitation.

dissociation of the primary EGFR-Neu dimer. The data presented in Fig. 3*C* are consistent with the notion that Neu is strongly (trans) phosphorylated by the EGFR within the primary EGFR-Neu heterodimer, as evidenced by almost complete elimination of tyrosine phosphorylation by AG1478. Neu autophosphorylation, however, apparently occurs to a lesser extent, because AG879 shows a modest effect. Studies performed with A431 membranes containing EGFR, and insect cell-expressed Neu have also shown that although the EGFR can strongly trans-phosphorylate Neu, there is little or no Neu-catalyzed trans-phosphorylation of the EGFR.[3]

The data presented in Fig. 3*D* compare the results of co-immunoprecipitation of ErbB3 with Neu from PC12 cells treated with HRG *versus* cells treated with EGF. The HRG stimulation would lead to the formation of an ErbB3-Neu (primary) heterodimer, and as previously reported, this yields a doublet in the ErbB3 Western blots that reflected different tyrosine phosphorylation states of ErbB3 (26). The lower mobility (*upper band*) is exclusively found with p85 (26), leading to the suggestion that this represents a phosphorylated form of ErbB3 that ultimately dissociates from Neu and forms a complex with p85. EGF induces the formation of a Neu-ErbB3 secondary dimer. In this case, the ErbB3 Western blot shows only a single band with a mobility essentially identical to the faster mobility ErbB3 band obtained in HRG-treated cells. Thus, these results demonstrate that the tyrosine phosphorylation of ErbB3 within Neu-ErbB3 heterodimers differs depending on whether it is a primary Neu-ErbB3 heterodimer or a secondary heterodimer, and presumably these differences will have important consequences regarding the specific substrates that are recruited to ErbB3.

There currently is little debate regarding the fundamental importance of growth factor-stimulated receptor dimer formation in the actions of members of the EGFR family or in the activities of a variety of other receptor tyrosine kinases. Recent studies have shown that receptor activation and dimerization are dependent on the ligand type (24) and that receptor dimerization is not a random process but is subjected to a certain hierarchy (16, 24). The data presented in this study now provide evidence that growth factors can actually stimulate a cascade of receptor dimerization events through a mechanism that reflects the enzyme-substrate nature of the interactions between members of the EGFR family. We propose that growth factor-stimulated receptor dimerization represents a transient rather than a stable interaction. The trans-phosphorylation of one receptor (*i.e.* the substrate) by its partner receptor (the enzyme) results in the dissociation of the receptor dimer (sim-

[3] P. Guy and K. Carraway, unpublished data.

ilar to the dissociation of an enzyme-product complex). This enables the individual, trans-phosphorylated receptor(s) to form new receptor dimers and to phosphorylate new partners. Table III shows that a variety of different primary and secondary receptor dimer combinations are possible, depending on the activating ligand used and the immunoprecipitating antibody. In the present work, we show that treatment of PC12 cells with HRG leads not only to the formation of an ErbB3-Neu primary receptor dimer but also to a Neu-EGFR secondary dimer and that treatment with EGF induces both a primary EGFR-Neu heterodimer and a secondary Neu-ErbB3 heterodimer.

The idea that primary receptor dimerization can give rise to secondary dimerization events is supported by various other lines of study. As alluded to above, data obtained from HRG-treated PC12 cells were consistent with the idea that the primary (HRG-stimulated) receptor dimer formed between ErbB3 and Neu was of a transient nature and upon dissociation leads to ErbB3-p85 interactions (26). A study of the reversible dimerization of the EGFR has shown that phosphorylated monomers appeared after receptor dimerization (19). This delayed appearance of phosphorylated EGFR monomers (as compared with the formation of phosphorylated dimers) could be attributed to the dissociation of the EGFR homodimer. Recent studies have also yielded data consistent with the idea that different members of the EGFR family can become phosphorylated and activated in a ligand-independent manner, *i.e.* in a manner somewhat analogous to the ligand-independent formation of secondary dimerization events between a phosphorylated/activated receptor monomer and a nonphosphorylated/inactive receptor monomer. Specifically, it has recently been shown that both the EGFR and Neu can be phosphorylated and activated (independent of EGF or HRG) by ligands for G protein-coupled receptors, presumably through a mechanism where a Src-like tyrosine kinase (activated downstream from the G protein-coupled receptor) binds and phosphorylates the EGFR or Neu (35). The secondary dimer formation is also in concert with the findings that Neu is subjected to trans-activation by both EGF and HRG receptors (36–38) as well as with the concept of lateral signaling within the cell membrane by combinatorial receptor interactions (16).

The ability to undergo both primary and secondary receptor dimerization events would provide a means by which each member of the EGFR family could form a heterogeneous population of phosphorylated receptor species (depending on the types of primary and secondary receptor dimers that are generated), thereby increasing the diversity of signaling pathways that can be initiated by a single growth factor. In addition, the signals induced by secondary receptor dimers will affect cells that have already been subjected to the first wave of signals emanating from the primary receptor dimerization events. Thus, it is conceivable that the second wave of signals might stimulate or arrest and elongate or terminate the effects induced by the first wave of signals. Thus, the mechanism of secondary dimerization provides an additional means for control and for fine tuning of signals initiated by the members of the EGFR family.

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The Pleckstrin Homology Domain Mediates Transformation by Oncogenic Dbl through Specific Intracellular Targeting*

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The pleckstrin homology (PH) domain is an ~100 amino acid structural motif found in many cellular signaling molecules, including the Dbl oncoprotein and related, putative guanine nucleotide exchange factors (GEFs). Here we have examined the role of the Dbl PH (dPH) domain in the activities of oncogenic Dbl. We report that the dPH domain is not involved in the interaction of Dbl with small GTP-binding proteins and is incapable of transforming NIH 3T3 fibroblasts. On the other hand, co-expression of the dPH domain with oncogenic Dbl inhibits Dbl-induced transformation. A deletion mutant of Dbl that lacks a significant portion of the PH domain retains full GEF activity, but is completely inactive in transformation assays. Replacement of the PH domain by the membrane-targeting sequence of Ras is not sufficient for the recovery of transforming activity. However, subcellular fractionations of Dbl and Dbl mutants revealed that the PH domain is necessary and sufficient for the association of Dbl with the Triton X-100-insoluble cytoskeletal components. Thus, our results suggest that the dPH domain mediates cellular transformation by targeting the Dbl protein to specific cytoskeletal locations to activate Rho-type small GTP-binding proteins.

The cytoskeletal-associated Dbl oncoprotein transforms NIH 3T3 cells (1) by activation of signaling pathways involving Rho-type GTP-binding proteins (2). Proto-Dbl is a 115-kDa cytoskeletal-associated protein that is found in brain, adrenal glands, and gonads (1). Oncogenic activation occurs as an outcome of an amino-terminal truncation of proto-Dbl, where a recombination event fuses about 10 kDa of an unidentified human gene product (from chromosome 3) on to the carboxyl-terminal half of Dbl (residues 498–925). The oncogenic Dbl protein contains at least two putative signaling motifs. The first is a region of 176 amino acids (residues 498–674) that was originally found to share significant homology with the *Saccharomyces cerevisiae* cell-division-cycle protein, Cdc24, and the breakpoint cluster region protein, Bcr[1] (3). This region, referred to as the Dbl homology (DH) domain, has been shown to be essential both for the transformation activity of oncogenic Dbl and for its ability to act as a GEF by stimulating the guanine nucleotide exchange activity of Cdc42Hs (4, 5). The second putative signaling motif is the pleckstrin homology (PH) domain (6, 7) and includes residues 703–812. Although PH domains appear to be relatively poorly conserved, both NMR and x-ray crystallographic studies of the PH domains of pleckstrin, dynamin, and spectrin indicate that they adopt a common three-dimensional structural motif (8–11).

Over the past few years, a growing family of oncogene products and other growth regulatory proteins have been shown to contain a DH domain in tandem with a PH domain. In addition to Cdc24 and Bcr, these include the Vav, Ost, Ect-2, Lbc, Lfc, and Dbs oncoproteins (12–17) and the activators of the Ras proteins, Sos (18), and Ras-GRF (19). All indications from previous studies are that the DH domain will form a binding site and in many cases contain GEF activity for Rho-like GTP-binding proteins (8, 9, 13, 14, 20–22). However, less is known about the roles of the PH domains. In the present study, we have used the Dbl oncoprotein as a model to examine the role of the PH domain in cellular transformation and GEF activity.

EXPERIMENTAL PROCEDURES

cDNA Transfection Studies—Transfection assays were done on duplicate cultures by adding 0.001, 0.01, 0.1, and 1 μg of DNA to the recipient NIH 3T3 cells using the Ca^{2+}-phosphate precipitation method (3). Foci (focus forming units) were scored 14 days after transfection, and the results were calculated as number of foci/pmol of DNA. The results listed in Fig. 1 and shown in Fig. 2*C* are the mean values of three transfection assays. Growth in soft agar was examined as described by Ron *et al.* (3).

Cellular Fractionation Studies—Control NIH 3T3 and different NIH 3T3 transfectants were lysed and fractionated into cytosolic (S), Triton X-100-solubilized membrane fractions (T), and Triton X-100-insoluble fractions (I) as described by Graziani *et al.* (1). Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 h at 37 °C. Specific Dbl products were detected by immunoprecipitation using anti-Dbl-2 antibodies (3), electrophoresed through a 12% polyacrylamide gel and autoradiographed. For the detection of the PH domains (*e.g.* Figs. 2*B* and 4*D*, below), cells were immunoprecipitated with anti-Flag M5 antibodies and electrophoresed through a 15% polyacrylamide gel followed by immunoblotting with anti-Flag M5 antibodies.

Measurements of GDP Dissociation from Cdc42Hs—The [^{3}H]GDP dissociation assays were carried out as described previously (4, 5). In Fig. 3*A*, the amounts of GST-Dbl and GST-DH (see Fig. 1) purified from Sf9 insect cells were estimated by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis. ~200 nM of GST-Dbl or GST-DH were incubated with 1 μg of RhoA protein preloaded with [^{3}H]GDP in 100 μl of reaction buffer at room temperature, and 16-μl aliquots were diluted into 5 ml of ice-cold termination buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 100 mM NaCl) at various time points. In Fig. 3*B*, 1 μg

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[1] The abbreviations used are: Bcr, break point cluster region protein; DH, Dbl homology; PH, pleckstrin homology; GAPs, GTPase-activating proteins for low molecular mass GTP-binding proteins; GRF, guanine nucleotide-releasing factor; GEFs, guanine nucleotide-exchange factor for low molecular mass GTP-binding proteins; GST, glutathione *S*-transferase.

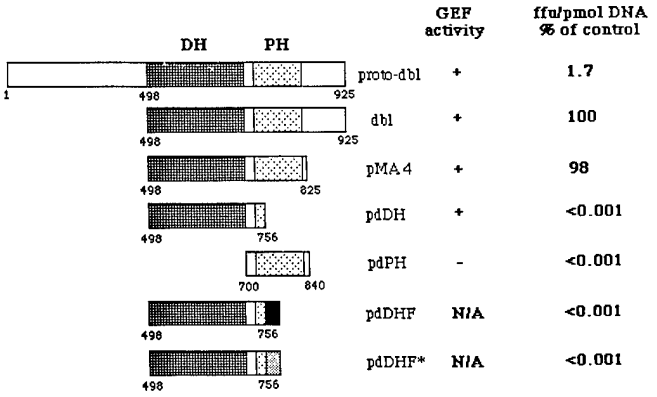

FIG. 1. **Schematic representations of oncogenic Dbl and different mutants of Dbl used in this study.** The abilities of these constructs to serve as GEFs for Rho and Cdc42Hs and to transform NIH 3T3 cells are summarized. pdDH represents the Dbl homology domain and pdPH is the pleckstrin homology domain. The abbreviation *ffu* represents focus forming units. 100% is 3×10^5 foci/pmol DNA. pDHF is a construct in which the PH domain sequences downstream from residue 750 are replaced with the carboxyl-terminal 16 amino acids of Ha-Ras, which include both the palmitylation and farnesylation sites (*black rectangle*). pdHF* is a construct encoding the DH domain of Dbl and the carboxyl-terminal 16 amino acids of Ha-Ras, except that the cysteine, which is normally farnesylated, has been changed to serine (*cross-hatched rectangle*). The GEF activity for these constructs has not been determined (*N/A*). The pdDH, pdDHF, and pdDHF* were subcloned from *dbl* by the polymerase chain reaction and inserted into the mammalian expression vector pZipneo. The GEF activities were measured as described (5) by the *in vitro* nitrocellulose filter binding assay either using the anti-Dbl immunoprecipitates from the NIH 3T3 transfectants (proto-Dbl, Dbl, and pMA4) or using the insect cell expressed peptides (DH and PH).

of [^{3}H]GDP-bound RhoA was incubated with 2 μM GST, 2 μM GST-PH (a fusion protein containing GST and the pleckstrin homology domain from the Dbl protein), 300 μM GST-Dbl, or 300 μM GST-Dbl and 2 μM GST-PH in a 100-μl reaction mixture.

RESULTS AND DISCUSSION

To investigate the role of the PH domain in cellular transformation mediated by the oncogenic Dbl protein, we analyzed several Dbl mutants for transforming activity in NIH 3T3 cells (Fig. 1). We found that while the transforming capability of a deletion mutant containing primarily the DH and PH domains (designated as *pMA4* in Fig. 1) was similar to that of the Dbl oncogene product, neither the DH domain nor the PH domain (*pdDH* and *pdPH*, respectively) alone showed any detectable effects on the growth of 3T3 fibroblasts. However, when Dbl and pdPH were co-expressed in NIH 3T3 cells (*dbl+pFlag/PHdbl*, Fig. 2*C*), we observed a significant reduction of the transforming activity by Dbl. Co-expression of Dbl with a Flag-tagged PH domain of the Dbl-related Vav oncoprotein (12), on the other hand, showed effects comparable with those obtained with the pFlag/neo vector control (Fig. 2*C*), even though it appeared to be more highly expressed (Fig. 2*B, lane 2*) than the PH domain of Dbl (Fig. 2*B, lane 1*). The level of expression of the Dbl oncoprotein was essentially equivalent in all cases (Fig. 2*A*).

To further confirm the selective inhibition of Dbl-induced transformation by the dPH domain, we used a second mammalian expression vector, pKH3 (23), to express the PH domain from either Dbl, Vav, or from the related yeast Cdc24 protein (20), together with the Dbl oncogene product. As shown in Fig. 2*C*, the expression of the dPH domain inhibited the focus-forming activity of oncogenic Dbl by ~40%, whereas co-expression of Dbl with the PH domain of Vav (pKH3/PHvav) had little effect. Mass populations of these transfected cells also were examined for their ability to display anchorage-independent growth. We observed that cells co-expressing Dbl and the dPH domain lost the ability to grow in soft agar (data not shown). In some cases, we found that expression of a Flag-tagged PH domain of Vav caused some inhibition of Dbl-induced growth in soft agar, suggesting that the Vav PH domain (perhaps when expressed at sufficient levels) was capable of competing with the PH domain of Dbl for a cellular target. However, it is likely that the Vav PH domain is a weak competitor, since we often observed no detectable effects with either the Flag-tagged protein or when expressing the PH domain of Vav from the pKH3 vector. We also have found no detectable effects on Dbl transformation when expressing the PH domain from Cdc24 (data not shown). Mass cultures of Dbl transfectants expressing the dPH domain also displayed a less transformed phenotype compared with Dbl transfectants alone or compared with cells co-expressing Dbl and the PH domain of Vav (data not shown). Taken together, these results suggest that the PH domain of Dbl behaves as a selective antagonist of Dbl-induced transformation, possibly by binding to a saturable and specific ligand in cells.

Previously we have shown that the DH domain alone is sufficient for the GEF activity for Cdc42Hs (5). Since oncogenic Dbl also stimulates the guanine nucleotide exchange activity of Rho, we examined whether the Dbl domain is sufficient for stimulating the activation of Rho. To do this, we compared Rho-GEF activities of approximately equal amounts (~200 nM) of insect cell-expressed, purified GST-Dbl and GST-DH domain. No significant differences were observed for the abilities of the GST-Dbl and GST-DH to stimulate [^{3}H]GDP dissociation from RhoA (Fig. 3*A*). These results suggest that the PH domain does not contribute to the GEF function of Dbl. This is further reinforced by the results in Fig. 3*B*, which show that the addition of excess *Escherichia coli* recombinant PH domain to GEF assay mixtures containing [^{3}H]GDP-bound RhoA and recombinant GST-Dbl has no detectable effect on the time-course of GST-Dbl-stimulated [^{3}H]GDP dissociation from RhoA. The GST-PH domain, alone, also shows no ability to stimulate [^{3}H]GDP dissociation from RhoA (compared with GST alone). Similar results were also obtained with [^{3}H]GDP-bound Cdc42Hs (data not shown). Thus, the dPH domain is not involved in the interactions of Dbl with RhoA and Cdc42Hs or in the direct regulation of the GEF catalytic activity of the DH domain.

The membrane association of βARK and spectrin has been attributed to their PH domains (24, 25). The PH domains of βARK, BTK, PLCγ, IRS-1, and Ras-GRF have been shown to bind to plasma membrane-associated $\beta\gamma$ subunits of the heterotrimeric G-proteins (26, 27), and they all behave as antagonists of G$\beta\gamma$-mediated signaling (28). Recent evidence also suggests that PH domains from many signaling molecules including βARK and Ras-GAP can bind to specific phospholipids, namely phosphatidylinositol 4,5-bisphosphate (PIP_2) (29). These findings raised the possibility that the PH domain mediates the membrane targeting of oncogenic Dbl. It has been shown that the introduction of a membrane-targeting sequence into the Ras GEFs, Cdc25 and Sos (30, 31), was sufficient to activate Ras, and more recently, that the addition of a membrane-targeting sequence in place of the PH domain of the Lfc oncoprotein was able to restore full transformation activity (32). Thus, we examined whether the substitution of the dPH domain with a membrane-targeting sequence enabled the DH domain of Dbl to induce transformation. A chimeric molecule containing the DH domain (residues 498–756) fused to the Ras membrane-targeting farnesylation signal sequence (designated *pdHF* in Fig. 1) was constructed and assayed for focus-forming activity in NIH 3T3 cells. This chimera was expressed at a comparable level to oncogenic Dbl and a percentage (10–20%) of the total chimeric molecules was targeted to the membrane

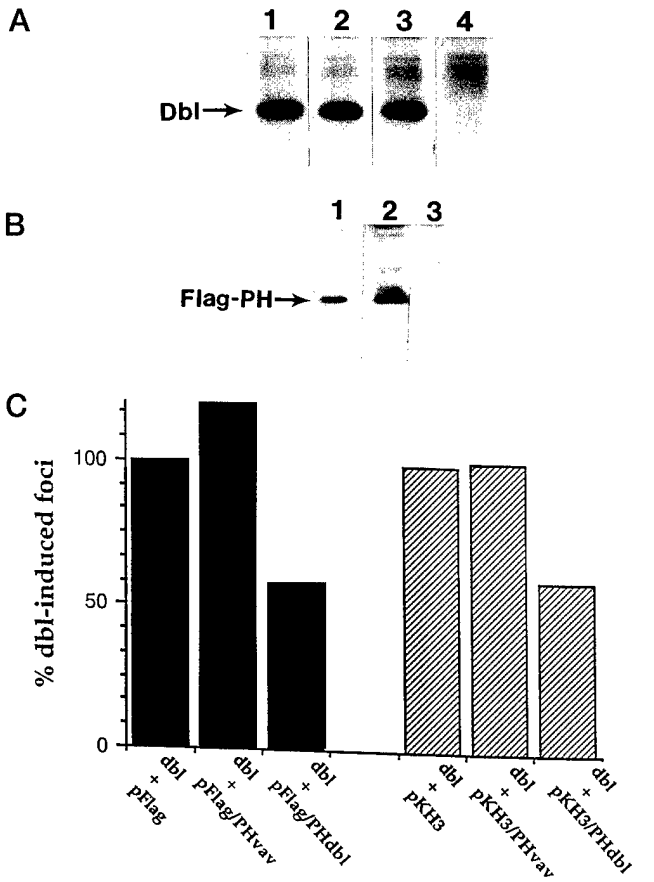

FIG. 2. **Expression of the PH domain inhibits Dbl-induced transformation in NIH 3T3 cells.** *A*, detection of the Dbl oncoprotein (using an anti-Dbl antibody) in NIH 3T3 transfectants. *Lane 1* represents cells expressing the Dbl oncoprotein. *Lane 2* represents cells co-expressing Dbl and the Dbl PH domain (dPH). *Lane 3* represents cells co-expressing Dbl and the Vav PH domain. *Lane 4* is a control, *i.e.* cells transfected with the plasmid (pFlag) used to express the PH domains. *B*, detection of the PH domains of Dbl and Vav (using M5 anti-Flag antibody) in NIH 3T3 cells. *Lane 1* represents cells co-expressing Dbl and the Dbl PH domain (dPH). *Lane 2* represents cells co-expressing Dbl and the Vav PH domain. *Lane 3* is a control (cells transfected with the pFlag plasmid). *C*, effects of the PH domains of Dbl and Vav on Dbl-induced foci-formation. pFlag/PHdbl and pKH3/PHdbl represent the mammalian expression vectors encoding the Dbl PH domain and pFlag/PHvav and pKH3/PHvav are the expression vectors encoding the Vav PH domain. The results shown represent the average of three independent experiments.

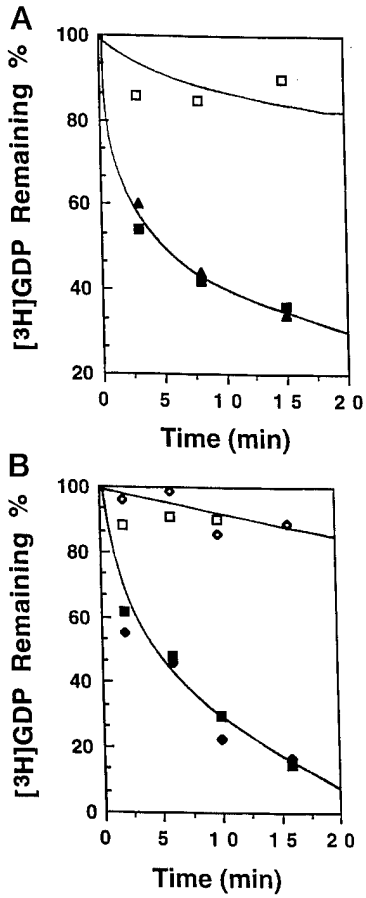

FIG. 3. **The PH domain is not directly involved in the regulation of the GEF activity of the Dbl oncoprotein.** *A*, comparison of the abilities of oncogenic Dbl (■) and the DH domain of Dbl (▲) to stimulate [^{3}H]GDP dissociation of RhoA. The dissociation of [^{3}H]GDP from RhoA, alone, is depicted by (□). *B*, effect of the PH domain on the kinetics of Dbl-stimulated [^{3}H]GDP dissociation from RhoA. ■ represents Dbl-stimulated GDP dissociation in the absence of the PH domain and ♦ represents Dbl-stimulated GDP dissociation in the presence of the PH domain. □ and ◇ represent the corresponding controls for RhoA in the absence of Dbl.

surface (*i.e.* the Triton X-100 solubilized fraction (*T*) in Fig. 4*A*). However, this did not restore transforming activity to the DH domain (Fig. 1). Although, one possible explanation is that the amount of the chimera expressed at the membrane surface was not sufficient to stimulate a transforming signal, this does not seem likely based on what we have observed regarding the range of expression of oncogenic Dbl that will yield cellular transformation (34).

We have reported previously that significant portions of both proto- and oncogenic Dbl are localized to the Triton X-100-insoluble fractions of transfected NIH 3T3 cells, suggesting an association with the cytoskeletal matrix (1). To address the possible role of the dPH domain in mediating this pattern of localization for the Dbl protein, stable transfectants of Dbl and Dbl deletion mutants (Fig. 1) were subjected to subcellular fractionation. The crude membrane fractions (P100) of the cells were solubilized either by 1% Triton X-100 or by treatment with 0.1% SDS and 0.25% sodium deoxycholate. Anti-Dbl immunoprecipitates revealed that a percentage of both the intact oncogenic Dbl protein and a truncation mutant pMA4 associated with the Triton X-100-insoluble fractions of cells (designated by *I* in Fig. 4, *B* and *C*). The amounts of oncogenic Dbl and pMA4 that were present in the Triton X-100-insoluble fraction typically varied between 50 and 70% of the total detectable protein, although in some cases (Fig. 4*B*) the percentage of oncogenic Dbl in this fraction was less than 50%. However, the DH domain of Dbl, which lacks transforming ability, was localized exclusively to the cytosolic fraction (designated *S* in Fig. 4*C*). When cells expressing the Flag-tagged PH domains were subjected to similar fractionation, the PH domains were found associated with the Triton X-100-insoluble fractions (Fig. 4*D*). These results suggest that the dPH domain is directly responsible for the association of oncogenic Dbl with the Triton X-100-insoluble cytoskeletal fraction and thus may serve to target the catalytic DH domain to the cytoskeleton.

We have reported previously that the DH domain is responsible for Dbl GEF function and is required for Dbl transforming activity (3, 5). Here, we demonstrate that while the dPH domain does not seem to be involved in the interactions of Rho-type small GTP-binding proteins with Dbl, it is essential for Dbl transforming activity. Thus, our present findings establish that both the DH and PH domains are required for the cellular function of Dbl. Indeed, the minimum structural unit (pMA4) of oncogenic Dbl conferring complete transforming activity just

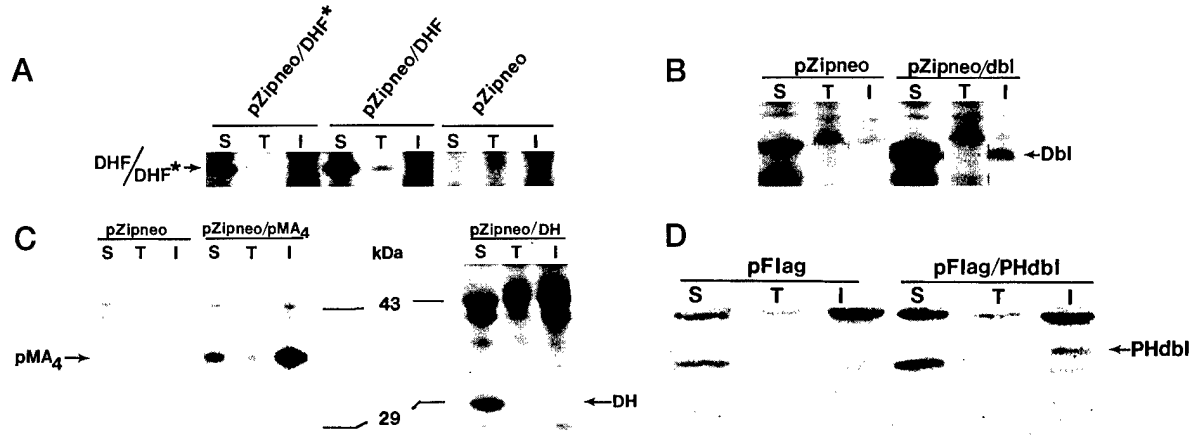

FIG. 4. **The PH domain mediates the cytoskeletal association of the Dbl oncoprotein.** *A*, the membrane attachment signal from Ha-Ras targets the DH domain to the Triton X-100-soluble fraction from cell membranes. pZIPneo/DHF (see Fig. 1) represents the construct encoding the DH domain of Dbl and the carboxyl-terminal 16 amino acids of Ha-Ras, including the palmitylation and farnesylation sites. pZIPneo/DHF* represents the construct encoding the DH domain of Dbl and the carboxyl-terminal 16 amino acids of Ha-Ras (except that the cysteine which serves as the farnesylation site has been changed to serine). *S* represents the soluble fraction, *T* is the Triton X-100-soluble fraction from membranes, and *I* is the Triton X-100-insoluble fraction. *B*, oncogenic Dbl is associated with the Triton X-100-insoluble fraction of cells. *C*, fractionations of the pMA4 and DH domain transfectants. *D*, fractionation of cells expressing the Dbl PH domain. The data shown in *A–C* were obtained by immunoprecipitating the Dbl proteins with the anti-Dbl antibody from cells that were labeled with [^{35}S]methionine and [^{35}S]cysteine. The data shown in *D* represent immunoblots using the anti-Flag M5 antibody.

encompasses the DH domain and the PH domain. The finding that plasma membrane-targeting of Dbl is not sufficient to confer transforming activity, coupled with the requirement of the dPH domain as the necessary and sufficient element for association of the Dbl protein with the Triton X-100-insoluble component, suggests that the function of the PH domain resides in its ability to target the catalytic DH domain to the cytoskeletal matrix. Whether this targeting function holds for other members of Dbl-related GEF family proteins remains to be seen. However, based on the observation that the PH domains of Dbl-related molecules Vav and Cdc24 do not act effectively as inhibitors of Dbl-induced transformation, it is an attractive possibility that different members of the family of Dbl-related proteins may be targeted by their PH domains to distinct cellular locations to activate various Rho-type GTP-binding proteins, in response to different extracellular stimuli such as epidermal growth factor, platelet-derived growth factor, lysophosphatidic acid, and bradykinin. This may also explain the finding that substitution of a membrane-targeting (*i.e.* Ras-farnesylation) sequence for the PH domain of Lfc restored its transformation capability (32), whereas this substitution did not restore transforming activity to a Dbl protein that just contains the DH domain. It may be that Lfc needs to be targeted to the plasma membrane to optimally couple to other protein components in its signaling pathway while Dbl needs to be targeted to a cytoskeletal location.

The identity of the ligand(s) that binds to the PH domain of oncogenic Dbl will represent an important focus of future studies. It seems likely, that given the hypervariable nature of the putative ligand-binding cleft in the PH domains that have thus far been identified (33), a complex diversity of ligands may exist that are responsible for mediating the actions of various PH domain-containing signaling molecules, including Dbl and related regulatory molecules of small GTP-binding proteins.

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Phosphatidylinositol 4,5-bisphosphate Provides an Alternative to Guanine Nucleotide Exchange Factors by Stimulating the Dissociation of GDP from Cdc42Hs*

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Members of the Rho subfamily of Ras-related GTP-binding proteins play important roles in the organization of the actin cytoskeleton and in the regulation of cell growth. We have shown previously that the *dbl* oncogene product, which represents a prototype for a family of growth regulatory proteins, activates Rho subfamily GTP-binding proteins by catalyzing the dissociation of GDP from their nucleotide binding site. In the present study, we demonstrate that the acidic phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), provides an alternative mechanism for the activation of Cdc42Hs. Among a variety of lipids tested, only PIP_2 was able to stimulate GDP release from Cdc42Hs in a dose-dependent manner, with a half-maximum effect at ~50 μM. Unlike the Dbl oncoprotein, which requires the presence of (free) guanine nucleotide in the medium to replace the GDP bound to Cdc42Hs, PIP_2 stimulates GDP release from Cdc42Hs in the absence of free guanine nucleotide. PIP_2, when incorporated into phosphatidylcholine carrier vesicles, binds tightly to the guanine nucleotide-depleted form of Cdc42Hs and weakly to the GDP-bound form of the GTP-binding protein but does not bind to GTP-bound Cdc42Hs, similar to what was observed for the Dbl oncoprotein. However, mutational analysis of Cdc42Hs indicates that the site that is essential for the functional interaction between PIP_2 and Cdc42Hs is distinct from the Dbl-binding site and is located at the positively charged carboxyl-terminal end of the GTP-binding protein. The GDP-releasing activity of PIP_2 is highly effective toward Cdc42Hs and Rho (and is similar to the reported effects of PIP_2 on Arf (Terui, T., Kahn, R. A., and Randazzo, P. A., (1994) *J. Biol. Chem.* 269, 28130–28135)), is less effective with Rac, and is not observed with Ras, Rap1a, or Ran. The ability of PIP_2 to activate Cdc42Hs (or Rho) and Arf provides a possible point of convergence for the biological pathways regulated by these different GTP-binding proteins and may be related to the synergism observed between Arf and Rho-subtype proteins in the stimulation of phospholipase D activity (Singer, W. D., Brown, H. A., Bokoch, G. M., and Sternweis, P. C. (1995) *J. Biol. Chem.* 270, 14944–14950).

The Rho subfamily of Ras-related GTP-binding proteins, which includes RhoA, Rac1, and Cdc42Hs, has been shown to regulate a diversity of cellular functions ranging from actin-mediated cytoskeletal rearrangements (Hall, 1994) to the stimulation of nuclear mitogen-activated protein kinases (Coso *et al.*, 1995; Minden *et al.*, 1995; Bagrodia *et al.*, 1995; Zhang *et al.*, 1995), transcription (Hill *et al.*, 1995), and DNA synthesis (Olson *et al.*, 1995). It is now well established that Rac1 is essential for growth factor-stimulated membrane ruffling and lamellipodia formation (Ridley *et al.*, 1992) and acts downstream from Ras in the stimulation of cell growth (Qui *et al.*, 1995), whereas RhoA controls the formation of stress fibers and focal adhesion complexes (Ridley and Hall, 1992). Cdc42 has been shown to be essential for bud-site assembly in *Saccharomyces cerevisiae* (Johnson and Pringle, 1990), for unidirectional and bidirectional cell growth in *Schizosaccharomyces pombe* (Miller and Johnson, 1994), and for filopodia formation in mammalian cells (Nobes and Hall, 1995; Kozma *et al.*, 1995). The macromolecular targets for the Rho subfamily GTP-binding proteins are just now beginning to be identified. Both GTP-bound Rac1 and Cdc42Hs bind to the M_r 85,000 regulatory subunit (p85) of the phosphatidylinositol 3-kinase (Zheng *et al.*, 1994a; Tolias *et al.*, 1995), to the p70 S6 kinase (Chou and Blenis, 1996), and to members of the p21-activated serine/threonine kinase (PAK) family (Manser *et al.*, 1994; Martin *et al.*, 1995; Bagrodia *et al.*, 1995). The GTP-bound form of RhoA also has been reported to stimulate phosphatidylinositol 3-kinase activity in platelets (Zhang *et al.*, 1993) as well as phosphatidylinositol 4-phosphate 5-kinase activity (Chong *et al.*, 1994). In addition, it recently has been shown that RhoA and Cdc42Hs can act synergistically with Arf to stimulate phospholipase D activity (Singer *et al.*, 1995).

Understanding how these different target activities are stimulated and then integrated to yield cytoskeletal changes and nuclear activities represents a formidable challenge. One approach is to determine how Rho subfamily GTP-binding proteins are activated, since this should represent the first key step in stimulating their target activities. One mode of activation of Rho-subtype GTP-binding proteins occurs through the stimulation of GDP dissociation (and consequently GTP/GDP exchange) by the family of Dbl-related proteins. The prototype for this family of guanine nucleotide exchange factors (GEFs)[1] is the Dbl oncoprotein, which was shown to act as a GEF for Cdc42Hs and RhoA (Hart *et al.*, 1991; Hart *et al.*, 1994). At present, 15 members of the Dbl-related family have been identified and characterized (Cerione and Zheng, 1996), with each member containing the characteristic Dbl homology domain in tandem with a Pleckstrin homology domain. Many of these

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[1] The abbreviations used are: GEF, nucleotide exchange factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(thiotriphosphate); PC, phosphatidylcholine.

proteins have been shown to have GEF activity including Cdc24 (Zheng *et al.*, 1994b), Ost (Horii *et al.*, 1994), Tiam-1 (Michiels *et al.*, 1995), and Lbc (Zheng *et al.*, 1995). Similar to the case for the interactions between heterotrimeric GTP-binding proteins (G proteins) and agonist-stimulated heptahelical receptors (Gilman, 1987), the Dbl-related GEFs bind preferentially to and stabilize the guanine nucleotide-depleted states of Rho-like GTP-binding proteins.

It is interesting that many of these Dbl-related proteins show a selective tissue distribution, whereas many of the Rho-subtype proteins (*e.g.* Cdc42Hs, RhoA, and Rac1) appear to be ubiquitous. This suggests that additional but as yet undiscovered Dbl-related proteins exist and/or that other mechanisms may be used to stimulate the activation of Rho-subtype GTP-binding proteins. In the present study, we describe one such potential alternative mechanism where the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is able to strongly stimulate GDP dissociation from Cdc42Hs and RhoA.

EXPERIMENTAL PROCEDURES

Materials—The small GTP-binding proteins Cdc42Hs, RhoA, Rac1, and Ha-Ras were expressed and purified as glutathione *S*-transferase (GST) fusion proteins from *Escherichia coli* as described previously (Hart *et al.*, 1994). The cDNAs encoding the K-Ras protein and Ras-GRF were kind gifts from Dr. Larry Feig (Tufts Medical School) and were expressed in *E. coli* as GST fusion proteins. The Rap1a protein was obtained from Dr. P. Polakis (Onyx Pharmaceutical, Emoryville, CA). The cDNA encoding Ran was the generous gift of Dr. M. Rush (New York University Medical Center, New York, NY). To express Ran in *E. coli* as a GST fusion protein, restriction sites for *Nco*I and *Hin*dIII were introduced immediately adjacent to the initiation and termination codons, respectively, using the polymerase chain reaction. The polymerase chain reaction product (730 base pairs) was then ligated with *NcoI/Hin*dIII-digested pGEX-KG (Pharmacia Biotech Inc.), and the resultant GST-Ran fusion protein was purified from transformed *E. coli* (JM101). Automated DNA sequencing revealed no mutations in the polymerase chain reaction product. The anti-Cdc42Hs polyclonal antibodies were raised against the unique carboxyl-terminal sequences of Cdc42Hs as described (Shinjo *et al.*, 1990). GST-Dbl protein was expressed in a baculovirus/Sf9 insect cell system (Hart *et al.*, 1994), and the Cdc42Hs-GTPase-activating protein was expressed and purified from *E. coli* (Barfod *et al.*, 1993). The ΔC7 Cdc42Hs truncation mutant was generated by polymerase chain reaction using the plaque-forming unit DNA polymerase (Stratagene), and the resulting sequences were verified through fluorescence automated sequencing. Lipids were purchased either from Sigma or from Avanti Polar Lipids. All lipids were dissolved in chloroform, dried under nitrogen, and resuspended by sonication in 50 mM Tris-HCl (pH 8.0) immediately prior to use. The pH was adjusted as necessary with NaOH. Radioisotope-labeled guanine nucleotides were obtained from DuPont NEN.

GDP Dissociation and GTP/GDP Exchange Assays—GDP dissociation and GTP/GDP exchange assays were carried out as described previously (Hart *et al.*, 1991). Two μg of Cdc42Hs loaded with [^{3}H]GDP was incubated with buffer mixtures containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ (buffer A) with various lipids or Dbl proteins for the indicated times at room temperature. Assays monitoring the dissociation of GDP were stopped by dilution (20-μl aliquots) into 10-ml ice-cold buffer A, and the protein-bound nucleotide was trapped by filtration on nitrocellulose filters. For GTP/GDP exchange assays, 1 mM GTP was also included in the reaction buffer.

GTPγS Binding Assays—Assays monitoring the dissociation of GTPγS from Cdc42Hs were performed as described above for GDP, except that [^{35}S]GTPγS was used, and the concentration of Cdc42Hs-GTPγS was ~0.4 μM. GTPγS binding was determined as in Hart *et al.* (1991). Two μg of GDP-bound Cdc42Hs were incubated in buffers containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10 μM [^{35}S]GTPγS (~5000 cpm/pmol), and 100 μM PIP_2, 0.5 μM GST-Dbl, or 100 μM phosphatidylcholine (PC) at 24 °C, and binding was determined at various time points.

Liposome·Protein Complex Formation Assays—Direct binding of liposome vesicles containing PIP_2 to Cdc42Hs was carried out by adapting the centrifugation protocol by Harlan *et al.* (1994). Briefly, Cdc42Hs was first loaded with GDP or GTPγS or depleted with nucleotide (Hart *et al.*, 1994). One μg of Cdc42Hs was then added to lipid vesicles (100 μl of total volume) with 500 μM carrier PC alone or 100 μM PIP_2 incorporated into the PC carrier vesicles through co-sonication. The mixture was incubated for 5 min before centrifugation in an ultraspeed Beckman airfuge (100,000 × *g*) for 30 min. The vesicles pelleted with this treatment were subjected to SDS-polyacylamide gel electrophoresis and anti-Cdc42Hs Western blot analysis, and the blot was visualized by the ECL method (DuPont NEN).

RESULTS AND DISCUSSION

The mode of regulation of Rho family GTP-binding proteins has been an area of intense research investigation (Boguski and McCormick, 1993) due to the involvement of these GTP-binding proteins in the stimulation of cytoskeletal changes and transcriptional activities as well as in the regulation of cell growth. In addition to protein factors that directly stimulate the guanine nucleotide exchange activities of Rho-subtype GTP-binding proteins, for which the Dbl oncoprotein is a prototype (Cerione and Zheng, 1996), various phospholipids have been implicated in the regulation of the Rac GTP-binding proteins through their effects on the GDP dissociation inhibitor molecule (Chuang *et al.*, 1993). In addition, the GTP-binding protein Arf, which undergoes GDP dissociation in response to PIP_2, appears to act synergistically with Rho-subtype proteins to stimulate (in a PIP_2-sensitive manner) phospholipase D activity (Malcolm *et al.*, 1994; Singer *et al.*, 1995; Moss and Vaughan, 1995). The latter finding is particularly interesting since we recently have found that Cdc42Hs is predominantly localized to Golgi membranes in mammalian cells and that its localization is influenced by different Arf mutants in a manner suggesting some type of interplay between Arf and Cdc42Hs.[2] Given these findings, we examined whether various phospholipids had any effect on the GTP-binding/GTPase cycle of Cdc42Hs.

Fig. 1*A* shows the results obtained when examining the effects of a panel of phospholipids on the rate of [^{3}H]GDP dissociation from Cdc42Hs. Only PIP_2 showed a significant stimulation of GDP dissociation from Cdc42Hs. Essentially no effect was observed when phosphatidic acid, phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol were incubated with [^{3}H]GDP-bound Cdc42Hs. We also have not detected any effects with inositol trisphosphate under conditions where PIP_2 strongly stimulates [^{3}H]GDP dissociation; however, PIP, at concentrations >500 μM, caused a slight increase in the rate of [^{3}H]GDP dissociation (data not shown).

Fig. 1*B* shows that the PIP_2-stimulated dissociation of GDP from Cdc42Hs is dose-dependent, with a half-maximal effect occurring at ~50 μM PIP_2. At PIP_2 levels ≥200 μM, the rate of GDP release from Cdc42Hs was increased ~10 fold.

The stimulation of GDP dissociation by PIP_2 was similar to that elicited by the oncogenic Dbl protein (Hart *et al.*, 1994). Fig. 2*A* shows time courses for the dissociation of [^{3}H]GDP from Cdc42Hs in the absence of activators or in the presence of the Dbl oncoprotein (~0.5 μM) or PIP_2 (100 μM). In these experiments, the dissociation of GDP was assayed in the presence of 1 mM GTP in the medium. Under these conditions, the half-time for [^{3}H]GDP dissociation from Cdc42Hs (in the presence of phosphatidylcholine as a control) was ~25 min, whereas Dbl-catalyzed GDP dissociation occurred with a half-time of ~1.5 min, and PIP_2-stimulated GDP dissociation occurred with a half-time of ~2.5 min.

It is interesting that when GDP dissociation was assayed in the absence of medium GTP, dramatic differences were observed between Dbl and PIP_2 (Fig. 2*B*). Specifically, PIP_2 was still capable of providing a strong stimulation of the initial rate of [^{3}H]GDP dissociation, whereas oncogenic Dbl failed to induce any detectable stimulatory effect.

These findings can be considered within the context of the

[2] Erickson, J. W., Zhang, C.-J., Kahn, R. A., Evans, T., and Cerione, R. A. (1996) *J. Biol. Chem.* **271,** in press.

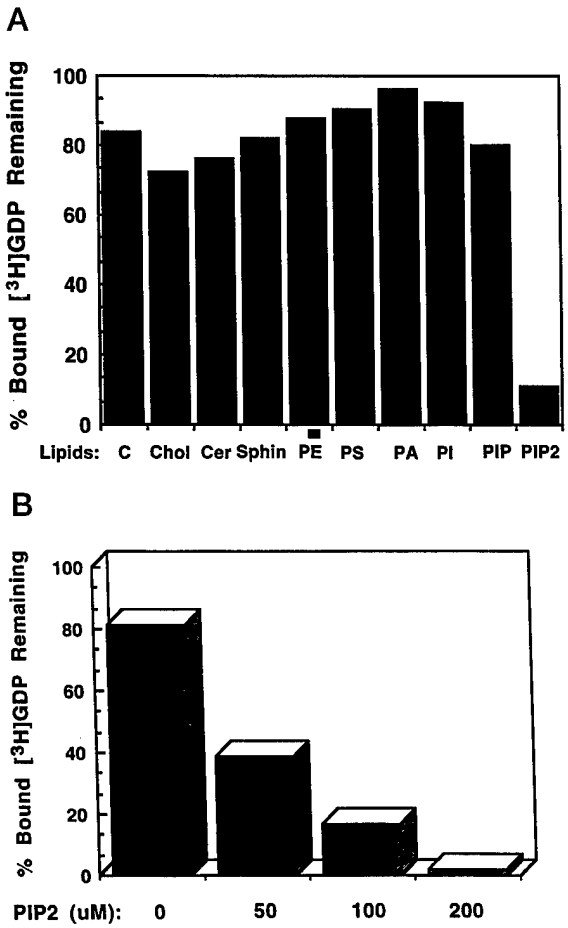

FIG. 1. **Effects of lipids on guanine nucleotide dissociation from Cdc42Hs.** In *A*, purified lipids at a concentration of 200 μM in buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, and 5 mM $MgCl_2$ were added to the incubation mixture with 2 μg *E. coli*-expressed [^{3}H]GDP-Cdc42Hs at a final concentration of 100 μM, and the reactions were terminated after 8 min at room temperature. *B*, dose-dependent [^{3}H]GDP dissociation from Cdc42Hs stimulated by PIP_2. GDP dissociation reactions were terminated 10 min after mixing PIP_2 and [^{3}H]GDP-bound Cdc42Hs.

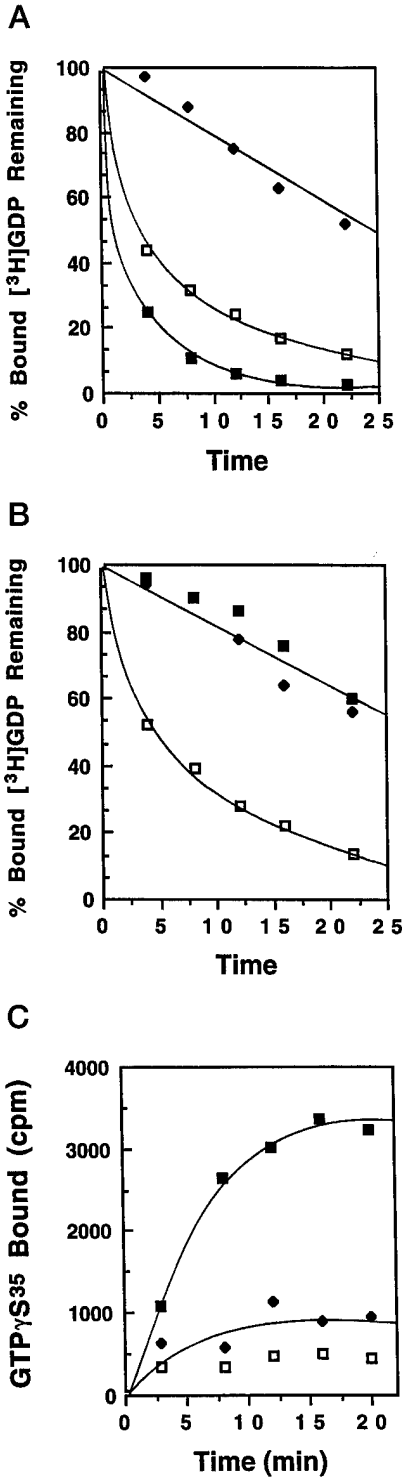

FIG. 2. **Comparisons of the guanine nucleotide exchange activities of Cdc42Hs stimulated by PIP_2 or the Dbl oncoprotein.** *A*, time courses of [^{3}H]GDP release from 2 μg Cdc42Hs stimulated by 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) under the GTP/GDP exchange assay conditions (with 1 mM free GTP). *B*, time courses of [^{3}H]GDP release from 2 μg Cdc42Hs stimulated by 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) in the absence of free nucleotides. *C*, effects of PIP_2 and Dbl on [^{35}S]GTPγS binding to Cdc42Hs. Cdc42Hs was preloaded with GDP, and the time courses for the binding of [^{35}S]GTPγS to Cdc42Hs in the presence of 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) were determined.

generally accepted model for the interactions of guanine nucleotides and GEFs with GTP-binding proteins. It typically has been assumed that GEFs act as antagonists of guanine nucleotide binding and that conversely, nucleotide binding weakens the interactions of GEFs with GTP-binding proteins. Thus, although Dbl weakens the affinity of GDP for Cdc42Hs, the amount of GDP present (*i.e.* initially bound to Cdc42Hs) is sufficient to maintain occupancy of the nucleotide site, even in the presence of this GEF. However, when a high excess of GTP is included in the medium, it effectively competes with GDP for the nucleotide site, thereby resulting in Dbl-catalyzed GTP-GDP exchange. This differs from the case for PIP_2, where this putative GEF is apparently more effective than Dbl in weakening the affinity of Cdc42Hs for guanine nucleotides. PIP_2 strongly stabilizes the guanine nucleotide-depleted state of Cdc42Hs; thus, it is difficult to observe a PIP_2-stimulated exchange of GDP for [^{35}S]GTPγS. In fact, under conditions where Dbl is able to elicit a strong stimulation of [^{35}S]GTPγS binding (when [GTPγS] = 0.5–1 μM), the addition of PIP_2 shows no detectable stimulation of GTPγS binding (Fig. 2*C*). In addition, although PIP_2 is more effective than Dbl in weakening the binding of guanine nucleotides to Cdc42Hs, it appears that guanine nucleotides also weaken the binding of PIP_2 to a greater extent than the binding of Dbl. Thus, although Dbl shows a weak but measurable stimulation of [^{35}S]GTPγS dissociation from Cdc42Hs, the addition of PIP_2 (at 50–100 μM) has no effect (data not shown).

Taken together, the results presented in Fig. 2 would suggest

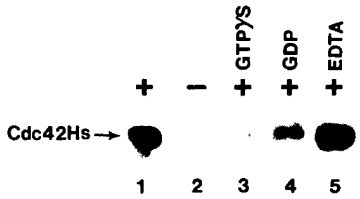

FIG. 3. **PIP_2 binds to the guanine nucleotide-depleted form of Cdc42Hs.** One μg of nucleotide-depleted Cdc42Hs (*EDTA lane*), Cdc42Hs bound to GDP (*GDP lane*), or Cdc42Hs bound to GTPγS (*GTPγS lane*) was incubated with 100 μM PIP_2 incorporated in PC vesicles for 10 min before ultracentrifugation in an airfuge. The resulting pellets were subjected to an anti-Cdc42Hs Western blot. *Lane −*, Cdc42Hs depleted of nucleotide incubated with PC vesicles alone; *lane +*, 0.1 μg Cdc42Hs as a positive control.

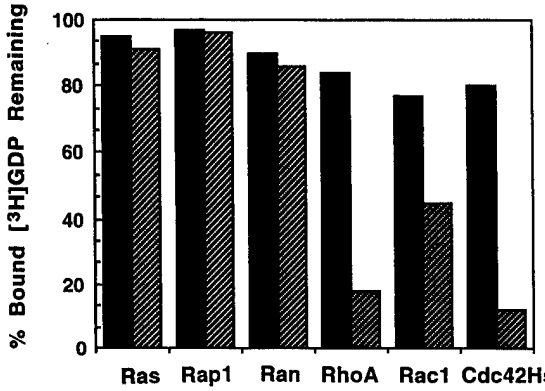

FIG. 4. **PIP_2 stimulates GDP dissociation from the Rho family GTP-binding proteins, Cdc42Hs and RhoA.** One μg of purified Ha-Ras, Rap1a, Ran, RhoA, Rac1, or Cdc42Hs were incubated with 100 μM PIP_2 under the GDP dissociation assay conditions for 8 min.

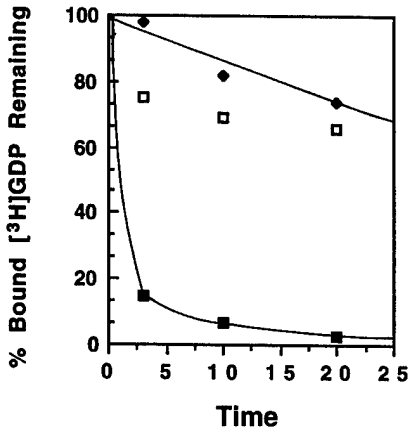

FIG. 5. **The carboxyl-terminal domain of Cdc42Hs is necessary for its interaction with PIP_2.** One μg of a carboxyl-terminal truncated (CΔ7) mutant of Cdc42Hs preloaded with [^{3}H]GDP was incubated with 100 μM PIP_2 (□), 0.5 μM GST-Dbl (■), or 1 μM GST (♦), and aliquots of the reaction mixtures were added to the termination buffers at the indicated time points.

that PIP_2 is capable of a direct interaction with Cdc42Hs and that this interaction is significantly influenced by the guanine nucleotide bound to the GTP-binding protein. Fig. 3 shows the results of such a direct binding experiment, using PC vesicles containing PIP_2. In this experiment, PC vesicles alone and PC vesicles containing PIP_2 were first incubated with Cdc42Hs, either in the guanine nucleotide-depleted state or in the GDP-bound state or the GTPγS-bound state, for 10 min at room temperature before pelleting the lipid vesicles by ultracentrifugation. Western blotting the pelleted vesicles with a specific anti-Cdc42Hs antibody revealed that Cdc42Hs, depleted of bound guanine nucleotide, was capable of tightly associating with the PC/PIP_2 vesicles, whereas Cdc42Hs containing bound GDP was capable of a weaker association with these vesicles. The GTPγS-bound Cdc42Hs showed no ability to associate with the PC/PIP_2 vesicles (*i.e.* relative to the background association observed with control PC vesicles). Thus, PIP_2 shows the same pattern of association with different guanine nucleotide-bound forms of Cdc42Hs as originally observed for the Dbl oncoprotein (Hart *et al.*, 1994).

It had been earlier reported that PIP_2 was able to stimulate the dissociation of [^{3}H]GDP from the Arf GTP-binding proteins (Terui *et al.*, 1994). Given our findings with Cdc42Hs, we were interested in determining whether PIP_2 might serve as a potential activator of other GTP-binding proteins, and in particular, other members of the Rho subfamily. As shown in Fig. 4, we found that PIP_2 was most effective on Cdc42Hs and RhoA, stimulating the dissociation of greater than 80% of the total bound [^{3}H]GDP within 5 min at room temperature. PIP_2 also was able to stimulate GDP dissociation from Rac1 (~50% of the total bound [^{3}H]GDP was dissociated after 5 min) but showed no ability to stimulate GDP dissociation from the Ha-Ras, Rap1a, or Ran GTP-binding proteins (Fig. 4), even when using PIP_2 levels as high as 0.5 mM. We also have found that PIP_2 will not stimulate [^{3}H]GDP dissociation from the K-Ras protein, under conditions where the Ras-GRF (Shou *et al.*, 1992) has a stimulatory effect (data not shown).

The ability of PIP_2 to regulate nucleotide binding to Rho subfamily proteins, as well as Arf, and to directly associate with Cdc42Hs (see Fig. 3) suggested that the Rho subfamily GTP-binding proteins must contain a specific PIP_2-binding site. Sequence comparisons between Rho subfamily GTP-binding proteins and two PIP_2-binding proteins, Gelsolin and Villin, indicated that the carboxyl-terminal domains of Cdc42Hs, RhoA, and the Rac proteins, which contain a number of basic amino acids, shared homology with amino acid residues 140–147 of Villin and 150–169 of Gelsolin. These regions of Villin and Gelsolin have been implicated in the binding of these proteins to PIP_2 (Janmey *et al.*, 1992). Thus, we constructed a deletion mutant of Cdc42Hs that lacked the carboxyl-terminal seven amino acids (including two arginines that were suspected to be involved in PIP_2 binding). This truncated Cdc42Hs molecule behaves like wild-type Cdc42Hs with regard to its intrinsic GTP-binding and GTPase activities and its ability to functionally couple to the Cdc42Hs-GTPase-activating protein (data not shown). Although it undergoes a slower rate of [^{3}H]GDP dissociation, compared to wild-type Cdc42Hs, it is still capable of interacting with Dbl and undergoing Dbl-catalyzed GDP dissociation (Fig. 5). However, the carboxyl-terminal truncated Cdc42Hs shows a markedly reduced response to PIP_2. These results then strongly argue that although Dbl and PIP_2 elicit similar effects (*i.e.* stimulation of GDP dissociation), they mediate these common effects from distinct binding domains on the GTP-binding protein.

In addition to serving as a precursor for the second messengers IP_3 and diacylglycerol (Berridge, 1993) and for the putative messenger PIP_3 (Stephens *et al.*, 1993), PIP_2 has been implicated as a regulator of the actin cytoskeleton, based on its ability to influence actin severing, capping, and bundling proteins *in vitro* (Janmey, 1994). Thus, it is interesting that in the present studies, we find that PIP_2 binds directly to and influences the nucleotide state of GTP-binding proteins that have been implicated in cytoskeletal regulation, *i.e.* Cdc42Hs and RhoA. However, these findings raise a number of important issues. One has to do with the mechanism by which PIP_2 stimulates GDP dissociation and how this compares with the mechanism by which Dbl stimulates GDP dissociation and guanine nucleotide exchange. Certainly the rate-limiting step in the activation of GTP-binding proteins, which occurs as an

outcome of the exchange of GTP for bound GDP, is the dissociation of the tightly bound GDP molecule. Both Dbl and PIP_2 strongly catalyze this dissociation event and stabilize the nucleotide-depleted state of the GTP-binding protein. Our data, in fact, would suggest that PIP_2 does this even more effectively than Dbl, such that PIP_2 can stimulate GDP dissociation from the nucleotide binding site of Cdc42Hs in the absence of added GTP, whereas Dbl cannot. It is possible that the differences exhibited by PIP_2 and Dbl reflect differences in the sites on Cdc42Hs (or related proteins) that bind these agents. At the present time, we know very little about the specific sites on Cdc42Hs that are responsible for binding Dbl, although mutations in Cdc42Hs that correspond to mutations in Ras that uncouple its binding to the GEF Sos (Mosteller *et al.,* 1994) do not uncouple Cdc42Hs from Dbl.[3] We would speculate at this point that a key conformational change that is necessary to loosen the binding of GDP to Cdc42Hs is induced by both Dbl and PIP_2 from distinct (binding) sites on the Cdc42Hs molecule. Future studies will be aimed at obtaining additional information regarding the conformational change in Cdc42Hs and related Rho subfamily proteins that is necessary for this rate-limiting step for activation.

A second key issue raised by these studies concerns whether, in fact, PIP_2 acts as a physiological regulator of Cdc42Hs and related proteins, and if so, how? It is tempting to speculate that the actin-regulatory activities of PIP_2 are related to the actions of Cdc42Hs and RhoA in mediating cytoskeletal changes such as filopodia formation and/or actin stress fiber formation. This is a particularly interesting possibility given the suggestions that a cascade of Rho subfamily GTPases (*i.e.* Cdc42Hs, Rac1, and RhoA) is operating in the regulation of cytoskeletal changes in certain cells (Nobes and Hall, 1995) and that a putative target for Cdc42Hs, the phosphatidylinositol 3-kinase (Zheng *et al.,* 1994a), which generates phosphatidylinositol compounds phosphorylated at the 3 position, may be upstream from Rac1 (Hawkins *et al.,* 1995; Nobes *et al.,* 1995). Thus, in addition to Dbl-related proteins, it is possible that phosphatidylinositol metabolites might serve as direct regulators of a signaling cascade that lead to changes in the actin cytoskeleton. It also is possible that phosphatidylinositol compounds work together (cooperatively) with Dbl-related proteins to activate GTP-binding proteins. We have not detected any type of cooperativity between oncogenic Dbl and PIP_2 in the stimulation of GDP dissociation from Cdc42Hs (data not shown). However, in some cases, Dbl-related proteins have been shown to bind to Rac without stimulating GDP dissociation (Miki *et al.,* 1993; Horii *et al.,* 1994), and in other cases, the presence of Dbl homology domains within proteins (*e.g.* the Ras-GEFs, Sos and Ras-GRF) have not yet been assigned a function (Shou *et al.,* 1992). Thus, it will be interesting to see if phosphatidylinositol compounds exert some type of regulatory or cooperative effect on the functions of these Dbl-related proteins. Finally, studies with purified phospholipase D indicate that the maximum activation of at least one isoform of the enzyme requires PIP_2, and the synergistic actions of the Arf GTP-binding protein (which also binds and is regulated by PIP_2 (Terui *et al.,* 1995)) and either RhoA or Cdc42Hs (Singer *et al.,* 1995). In the future, we intend to examine these possibilities further as well as determine just how PIP_2 levels in cells might be coordinated with the activation-deactivation cycle of Cdc42Hs and/or RhoA. For example, it is possible that signaling pathways that lead to an increase in PIP_2 levels will also promote the generation of guanine nucleotide-depleted Cdc42Hs and/or RhoA. Situations which then lead to a decrease in PIP_2 levels (*e.g.* stimulation of the hydrolysis of PIP_2 by phospholipase C enzymes) would then enable cellular GTP to bind to these GTP-binding proteins, thereby stimulating their activation. However, it also is possible that at the cellular levels of GTP (>10 μM), the exchange of GDP for GTP can occur even in the presence of high concentrations of PIP_2. Thus far, it has been difficult to test these levels of GTP in conventional GTP-binding assays; however, we hope in the future to be able to use fluorescence spectroscopic approaches to determine if, in fact, such a PIP_2-stimulated nucleotide exchange reaction is feasible.

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[3] Y. Zheng, unpublished results.

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Lfc and Lsc Oncoproteins Represent Two New Guanine Nucleotide Exchange Factors for the Rho GTP-binding Protein*

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Lfc and Lsc are two recently identified oncoproteins that contain a Dbl homology domain in tandem with a pleckstrin homology domain and thus share sequence similarity with a number of other growth regulatory proteins including Dbl, Tiam-1, and Lbc. We show here that Lfc and Lsc, like their closest relative Lbc, are highly specific guanine nucleotide exchange factors (GEFs) for Rho, causing a >10-fold stimulation of [^{3}H]GDP dissociation from Rho and a marked stimulation of GDP-[^{35}S]GTPγs (guanosine 5′-*O*-(3-thiotriphosphate) exchange. All three proteins (Lbc, Lfc, and Lsc) are able to act catalytically in stimulating the guanine nucleotide exchange activity, such that a single molecule of each of these oncoproteins can activate a number of molecules of Rho. Neither Lfc nor Lsc shows any ability to stimulate GDP dissociation from other related GTP-binding proteins such as Rac, Cdc42, or Ras. Thus Lbc, Lfc, and Lsc appear to represent a subgroup of Dbl-related proteins that function as highly specific GEFs toward Rho and can be distinguished from Dbl, Ost, and Dbs which are less specific and show GEF activity toward both Rho and Cdc42. Consistent with these results, Lbc, Lfc, and Lsc each form tight complexes with the guanine nucleotide-depleted form of Rho and bind weakly to the GDP- and GTPγS-bound states. None of these oncoproteins are able to form complexes with Cdc42 or Ras. However, Lfc (but not Lbc nor Lsc) can bind to Rac, and this binding occurs equally well when Rac is nucleotide-depleted or is in the GDP- or GTPγS-bound state. These findings raise the possibility that in addition to acting directly as a GEF for Rho, Lfc may play other roles that influence the signaling activities of Rac and/or coordinate the activities of the Rac and Rho proteins.

The Dbl family constitutes a group of oncoproteins and growth regulatory factors that have been implicated in a diversity of biological responses. Among the members of this family is the prototype Dbl oncoprotein, as well as Cdc24, a *Saccharomyces cerevisiae* cell-division-cycle protein involved in bud-site assembly (1, 2), Bcr, the breakpoint cluster region protein that has been implicated in the development of certain human leukemias (3), Tiam-1, which was first identified as a gene product involved in cell invasiveness and metastasis (4), and the *vav* (5), *ost* (6), *ect2* (7), *tim* (8), *fgd1* (9), *abr* (10), *dbs* (11), *lbc* (12), *lfc* (13), and *lsc* (14) oncogene products. The Dbl oncoprotein was first discovered when transfecting the DNA from diffuse B cell lymphomas into NIH 3T3 fibroblasts (1). Analysis of the primary amino acid sequence of the Dbl protein indicated that it contained a region of ~250 amino acids that shared homology with Cdc24 and Bcr. Given that genetic evidence placed Cdc24 upstream of Cdc42 in the bud-site assembly pathway in *S. cerevisiae*, it seemed plausible that Dbl regulated the actions of the human Cdc42 protein (Cdc42Hs). This led to the biochemical demonstration that Dbl is a guanine nucleotide exchange factor (GEF)[1] for Cdc42Hs and Rho (15, 16) and that Cdc24 is a highly specific GEF for the *S. cerevisiae* Cdc42 protein (Cdc42Sc) (2). It also was shown that the region of sequence similarity that Dbl shared with Cdc24 was critical for both GEF activity and for cellular transformation (16). More recent sequence analysis has subdivided this region of sequence similarity into two domains that are shared among all of the members of the Dbl family. The first domain, designated the Dbl homology (DH) domain, is essential for the GEF activity of Dbl, and the second domain, which shares homology with the platelet protein pleckstrin (designated the PH domain), is critical for the proper cellular targeting of Dbl and related proteins (13, 17).

Based on the initial biochemical studies performed on Dbl and Cdc24, it has been generally assumed that all proteins that contain a DH domain-PH domain in tandem will be GEFs for Rho-subtype proteins. In some cases this has been borne out. For example, Tiam-1 shows *in vitro* GEF activity toward Rac, Rho, and Cdc42 (18), and Lbc is a specific GEF for Rho (19). However, in a number of other cases, no GEF activity has yet been associated with the Dbl-related protein. This raises some important questions. 1) Are all DH domains involved in GEF activity or do they serve other biological roles, for example in the recruitment of Rho-related GTP-binding proteins to particular cellular locations and/or signaling complexes? 2) What accounts for the presumed specificity in the functional coupling of Rho-like proteins to Dbl-related molecules? In some cases *in vivo* specificity is probably mediated by cellular targeting, as accomplished by the individual PH domains of the different Dbl proteins. However, there also are clear indications that certain DH domains couple with high specificity to GTP-binding proteins, as exemplified by the interaction between Lbc and Rho. Overall, a better understanding of the regulation of Rho-re-

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; GST, glutathione *S*-transferase; JNK, c-Jun kinase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Mant, *N*-methylanthraniloyl; PH, pleckstrin homology.

lated GTP-binding proteins by members of the Dbl family is of broad significance, given the highly coordinated actions of the Rho proteins in mediating a series of cytoskeletal alterations including filopodia and lamellipodia formation and the generation of stress fibers (20–23), as well as in stimulating DNA synthesis (24–29) and promoting cell morphology changes and cell motility (30).

In the present study, we have begun to address the questions raised above concerning the functions of Dbl-related proteins. To do this we have taken advantage of what appears to be a subgroup within the Dbl family, for which the prototype is Lbc and which includes the recently identified Lfc and Lsc oncoproteins. Lfc and Lsc (*i.e.* the "first" and "second" cousins of Lbc) were initially identified along with Dbs ("Dbl's big sister"), as the products of cDNAs that induce transformation, by using a retroviral vector-based expression system to transfer a library of cDNAs from the murine 32D or B6SUtA$_1$ hematopoietic cell lines into NIH 3T3 fibroblasts (31). Both Lfc and Lsc share the highest sequence similarity with the Lbc oncoprotein, within the regions of the DH and PH domains. In this work, we first set out to determine if like Lbc, the Lfc and Lsc proteins were capable of stimulating guanine nucleotide exchange on Rho-related proteins. We also were interested in determining if Lfc and Lsc demonstrated a high degree of specificity in their binding and GEF activities. The demonstration that Lfc and Lsc, as well as Lbc, were highly specific in their interactions with Rho proteins could lead to new insights regarding the elements within DH domains that impart the ability to recognize individual members of the Rho family.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—The GST-Lsc and GST-Lfc proteins were prepared by first inserting the cDNAs encoding a fragment of Lfc (fragments 208–573) and Lsc (fragments 333–778), which encompasses the DH and PH domains of these proteins, into the pGEX2T vector, and then the glutathione *S*-transferase (GST) constructs were cloned into the *Bam*HI site of pAX142 (13, 14). The GST constructs were digested from pAX142 at the *MluI/SalI* sites; the fragments were then blunt-ended and inserted into the *MluI/Sma*I sites of the baculovirus transfer vector pVL1393. *Spodoptera frugiperda* cells (SF21) were infected with recombinant baculovirus; the cells were collected at 48 h postinfection and lysed in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The GST fusion proteins were purified by glutathione-agarose affinity chromatography. Preparation of GST-Cdc24 and GST-Lbc and their expression in SF21 cells has been previously described (2, 19) as has the expression of GST-GRF in *Escherichia coli* (32). The GTP-binding proteins RhoA, Rac, Cdc42, and Ras were expressed as His-fusion proteins in *E. coli*. The construction of an expression vector containing GST fused to the cDNAs encoding the full-length genes for different Ras-related GTP-binding proteins has been described (16, 19). For RhoA, Rac, Ras, and Cdc42, the cDNAs were transferred into the *Bam*HI/*Eco*RI sites of a modified pET15b vector that allowed the coding region to be in frame with the upstream hexa-His tag. The plasmid was transformed into BL21 (DE3) *E. coli*, and an overnight culture from a single colony was used to inoculate a 1-liter culture that was grown at 37 °C, while shaking, to an A_{560} of 0.6 (this took approximately 4 h). At this time, the protein expression was induced by the addition of 200 μM isopropyl-β-D-thiogalactoside for 2 h. Bacteria were harvested by centrifugation and frozen at −80 °C. The pellets were thawed in lysis buffer consisting of 20 mM Tris-HCl (pH 8.0), 5 mM imidazole, 500 mM NaCl, 1 mM sodium azide, 200 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, and resuspended using a glass/Teflon Dounce homogenizer. The bacteria were lysed on ice by adding 0.5 mg/ml lysozyme with 5 mM EDTA followed by 10 μg/ml DNase I (Boehringer Mannheim) and $MgCl_2$ to 10 mM. The lysate was cleared by centrifugation for 30 min at 30,000 rpm. The proteins were purified by affinity chromatography, using an iminodiacetic acid agarose column, charged with $NiSO_4$. The 17-amino acid hexa-His tag was then removed by thrombin cleavage. In all assays, the GST fusion proteins were used without the removal of GST. The amounts of the GEFs used in all experiments were estimated by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis.

GDP/GTP Exchange Assays—The GDP dissociation and GTP binding assays were carried out by the filter binding method at 24 °C as described previously (15). The quantities of GTP-binding proteins and the amounts of GST, GST-Lsc, GST-Lfc, GST-Lbc, GST-Cdc24 and GST-GRF used for each individual experiment are indicated in the figure legends. In the initial screens to detect guanine nucleotide exchange activity, the GTP-binding proteins were loaded with [^{3}H]GDP and incubated with control and test proteins. After 15 min, the samples were quenched with ice-cold dilution buffer, containing 10 mM $MgCl_2$, and collected by filter binding and counted to determine the relative amount of bound [^{3}H]GDP remaining. To further characterize potential nucleotide exchange activities detected in the initial screen, a full time course of [^{3}H]GDP dissociation and [^{35}S]GTPγS binding was performed.

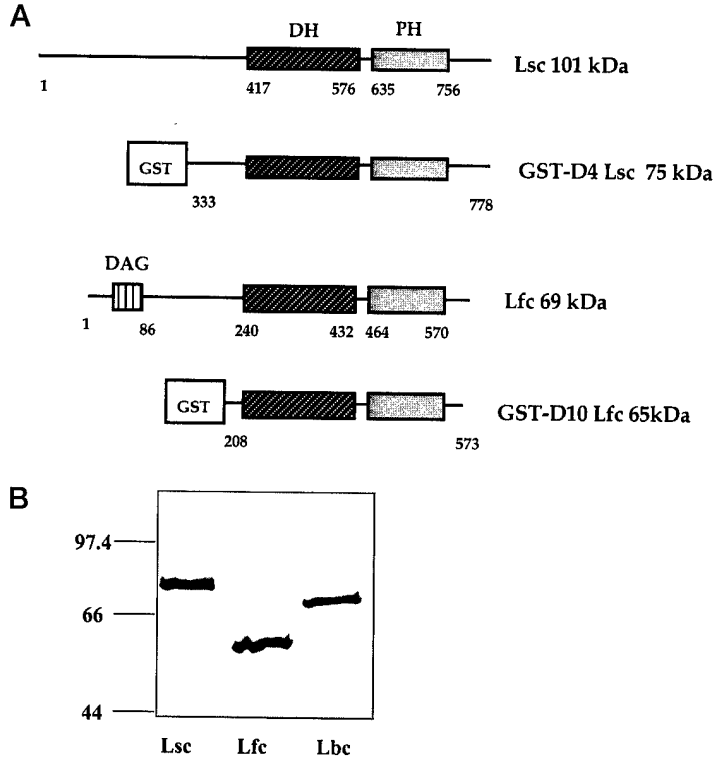
Fluorescence Spectroscopy—Fluorescence measurements were made using an SLM 8000C spectrofluorimeter in the photon counting mode. Samples were stirred continuously and thermostated at 25 °C in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$. *N*-Methylanthraniloyl (Mant)-dGTP was synthesized according to the published procedure (33) from dGTP and *N*-methylisatoic acid (Molecular Probes, Eugene OR). Guanine nucleotide exchange assays were carried out by initially incubating 650 nM RhoA with 450 nM Mant-dGTP and monitoring Mant fluorescence (excitation = 350 nm, emission = 440 nm). Exchange of Mant-dGTP for GDP on RhoA was then initiated by the addition of either GST-Lbc, GST-Lfc, or GST-Lsc, so that the final GEF concentration varied between 25 and 100 nM. 200 s after nucleotide exchange was initiated, EDTA was added to a final concentration of 6.7 mM, thus allowing the exchange of Mant-dGTP for GDP on RhoA to be driven to completion; this was done to demonstrate that equal amounts of RhoA bound to Mant-dGTP were present in each sample. The initial rates for the nucleotide exchange activities catalyzed by Lbc, Lfc, or Lsc were estimated by applying linear fits to the first 50 s after the addition of the GEF, using IgorPro wavemetrics software.

Complex Formation of His-GTP-binding Proteins with GST-Lsc and GST-Lfc—Interactions between G-proteins and GEFs were detected *in vitro* by using immobilized GST-Lsc and GST-Lfc, and as positive controls, GST-Lbc, GST-Cdc24, and GST-GRF were bound to glutathione-agarose beads to co-precipitate purified His-tagged (clipped) GTP-binding proteins. Initially all interactions were assayed using the nucleotide-free state of the GTP-binding proteins. Co-precipitations were performed in 500-μl volumes of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (incubating for 2 h at 4 °C). The agarose beads were washed in the precipitation buffer three times by centrifugation and resuspended in Laemmli buffer, subjected to SDS-polyacrylamide gel electrophoresis, and Western blotted. Co-precipitations were performed for each GTP-binding protein individually, and interactions were detected using anti-RhoA monoclonal antibody (Santa Cruz Biotechnology), or anti-Rac polyclonal antibody (Santa Cruz Biotechnology), anti-Ras monoclonal antibody (Santa Cruz Biotechnology), or anti-Cdc42 antibody (raised against the carboxyl-terminal 23 amino acids), by the ECL method (Amersham Corp.).

In order to fully characterize the nucleotide dependence of the interactions occurring between GEFs and GTP-binding proteins in the nucleotide-free state, we repeated co-precipitations using RhoA and Rac bound to GDP and GTPγS. These experiments were performed as described above except that the GTP-binding proteins were preloaded with the appropriate nucleotide, and the EDTA was replaced with 10 mM $MgCl_2$ in order to stabilize nucleotide binding to the GTP-binding proteins.

RESULTS

The principal aim of these studies was to determine whether the Lfc and Lsc proteins were capable of functionally coupling to the members of the Rho subfamily of GTP-binding proteins. Previous studies have shown that the DH domain of Dbl is essential for both its GEF activity and transforming capability (16). All members of the Dbl family also contain a PH domain, which is immediately carboxyl-terminal to the DH domain, and recent findings suggest that the PH domain is important for cellular targeting rather than for GEF activity (13, 17). However, because of the possibility that the PH domains of Dbl proteins may have other regulatory functions, and because we have found that the presence of surrounding sequences includ-



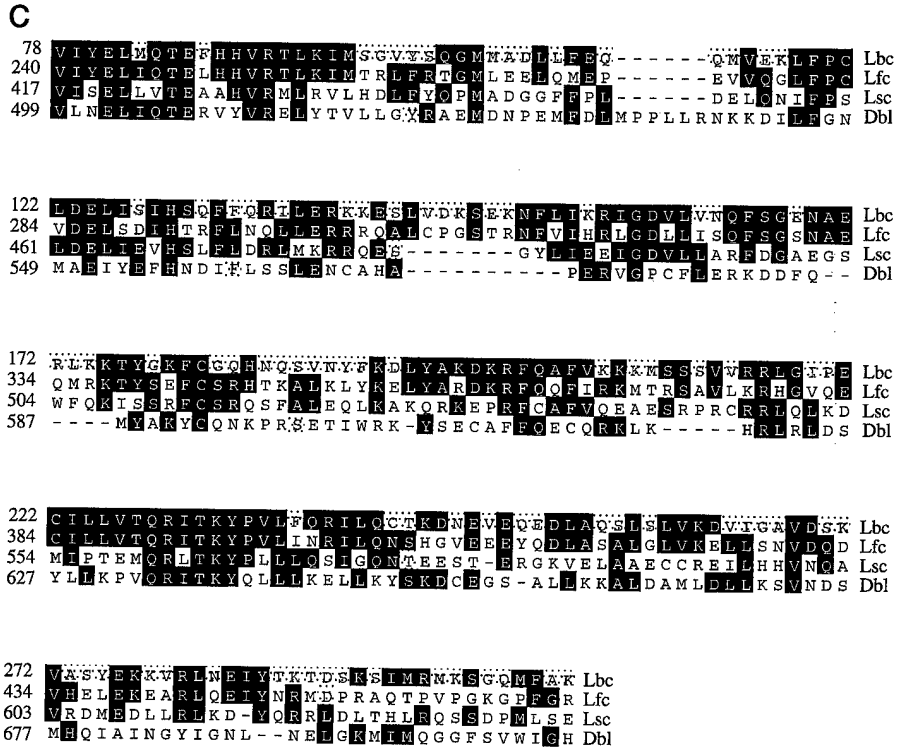


FIG. 1. **Expression of Lfc and Lsc as GST fusion proteins.** *A*, schematic representation of full-length Lfc and Lsc and the GST-Lfc and GST-Lsc fusion proteins that were expressed and assayed for GEF activity. D4 Lsc and D10 Lfc refer to constructs that have been described previously (13, 14). *B*, expression and purification of Lfc, Lsc, and Lbc as GST fusion proteins. SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) of the purified GST fusion proteins which were prepared from SF21 insect cell lysates infected with recombinant viruses that encode the Lfc, Lsc, and Lbc proteins. *C*, comparison of the Dbl homology domains from Lbc, Lfc, Lsc, and Dbl. Alignment of residues 78–304 of Lbc to similar sequences of Lfc, Lsc, and Dbl is shown. *Boxed* residues represent sequence identities.

ing the PH domain may be necessary to achieve the proper folding of DH domains, we expressed and purified forms of the Lfc and Lsc proteins that include both the DH and PH domains, as well as some additional flanking sequences. Fig. 1*A* shows the schematic representations of both the full-length Lfc and Lsc proteins and the glutathione *S*-transferase (GST) fusion proteins that were expressed in *S. frugiperda* (SF21) cells and assayed for GEF activities (see below). Fig. 1*B* shows the SDS-polyacrylamide gel electrophoretic profiles of the GST-Lfc and GST-Lsc proteins. Both of the proteins could be highly purified by glutathione-agarose chromatography and appeared to be fully soluble.

Determination of Guanine Nucleotide Exchange Activity for Lfc and Lsc—Comparisons of the Dbl domains of Lfc and Lsc with other members of the Dbl family show that these domains are most similar to those for Lbc (Fig. 1*C*). Given that Lbc is a highly specific GEF for Rho, we examined whether the Lfc and Lsc proteins were capable of similar biochemical activities. Fig. 2, *A* and *B*, shows that this is the case. Both Lfc (Fig. 2*A*) and Lsc (Fig. 2*B*) were highly effective in stimulating the dissociation of [^{3}H]GDP from *E. coli*-expressed RhoA. In the absence of any regulatory protein, the half-time for the dissociation of

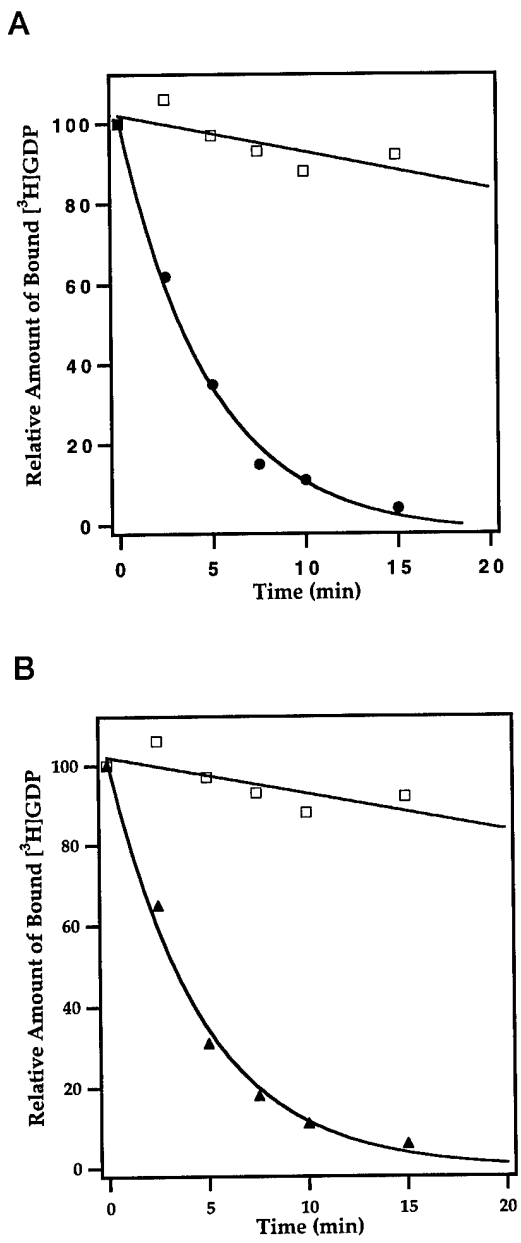


FIG. 2. **Stimulation of GDP dissociation from RhoA by Lfc and Lsc.** *A*, measurement of the dissociation of [^{3}H]GDP from the *E. coli* expressed RhoA protein as stimulated by GST-Lfc. *B*, measurement of the dissociation of [^{3}H]GDP from the *E. coli* expressed RhoA protein as stimulated by GST-Lsc. For each experiment, 2 μg of recombinant RhoA protein were preloaded with [^{3}H]GDP and then added to incubations containing 5 μg of GST (□), 1 μg of GST-Lfc (●), or 1 μg of Gst-Lsc (▲) in reaction buffer containing 100 μM cold GTP for the indicated time before terminating the reactions by the nitrocellulose filter binding method (see "Experimental Procedures").

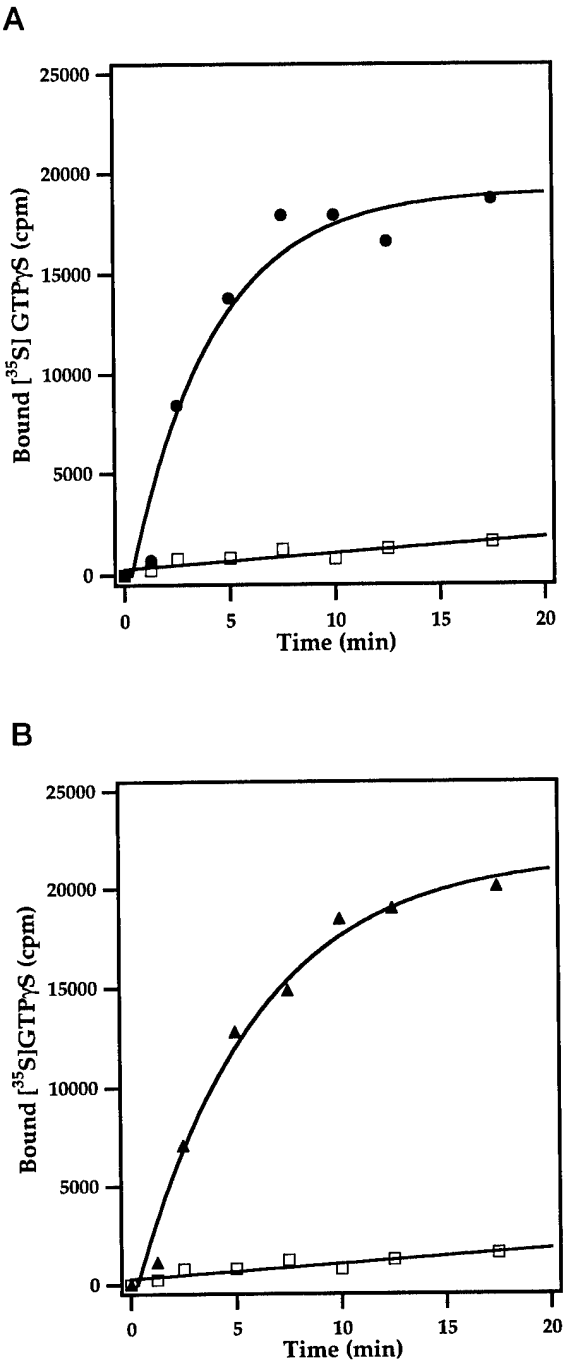


FIG. 3. **Stimulation of GTPγS binding to RhoA by Lfc and Lsc.** *A*, measurement of GST-Lfc-stimulated GTPγS binding to *E. coli* expressed RhoA protein. *B*, measurement of GST-Lsc-stimulated GTPγS binding to *E. coli* expressed RhoA protein. For each experiment, 5 μg of GST (□), 1 μg of Gst-Lfc (●), or 1 μg of GST-Lsc (▲) were added to 2 μg of GDP-bound RhoA in a reaction mixture containing [^{35}S]GTPγS for the indicated time before termination of the reactions by the nitrocellulose filter binding method.

GDP from RhoA is relatively slow, *i.e.* >30 min at room temperature. However, both Lfc and Lsc proteins were able to markedly accelerate the rate of GDP dissociation by at least 10-fold such that the half-time for GDP dissociation was 2–3 min in the presence of these regulatory proteins.

Fig. 3, *A* and *B*, shows that the Lfc and Lsc proteins also strongly stimulated the exchange of GDP for [^{35}S]GTPγS. Here again in the absence of any regulatory factor, RhoA is capable of little if any guanine nucleotide exchange over a period of 20 min. However, both Lfc and Lsc catalyzed the complete exchange of GDP for GTPγS within ~10 min, thus indicating that these proteins qualify as effective GEFs for the RhoA protein.

We have closely compared the initial rates of guanine nucleotide exchange on RhoA that are catalyzed by Lfc, Lsc, and Lbc, under conditions where RhoA was present in at least a 6-fold excess over the GEFs, using a very sensitive fluorescence spectroscopic assay (Fig. 4*A*). This assay is based on the finding that the fluorescence emission of Mant-GTP is enhanced upon binding to GTP-binding proteins. Thus, under conditions where guanine nucleotide exchange is catalyzed, the presence of Mant-dGTP in a cuvette solution containing RhoA will result in an exchange of the GDP that was originally bound to RhoA for the Mant-nucleotide and thereby provide a real-time assay for

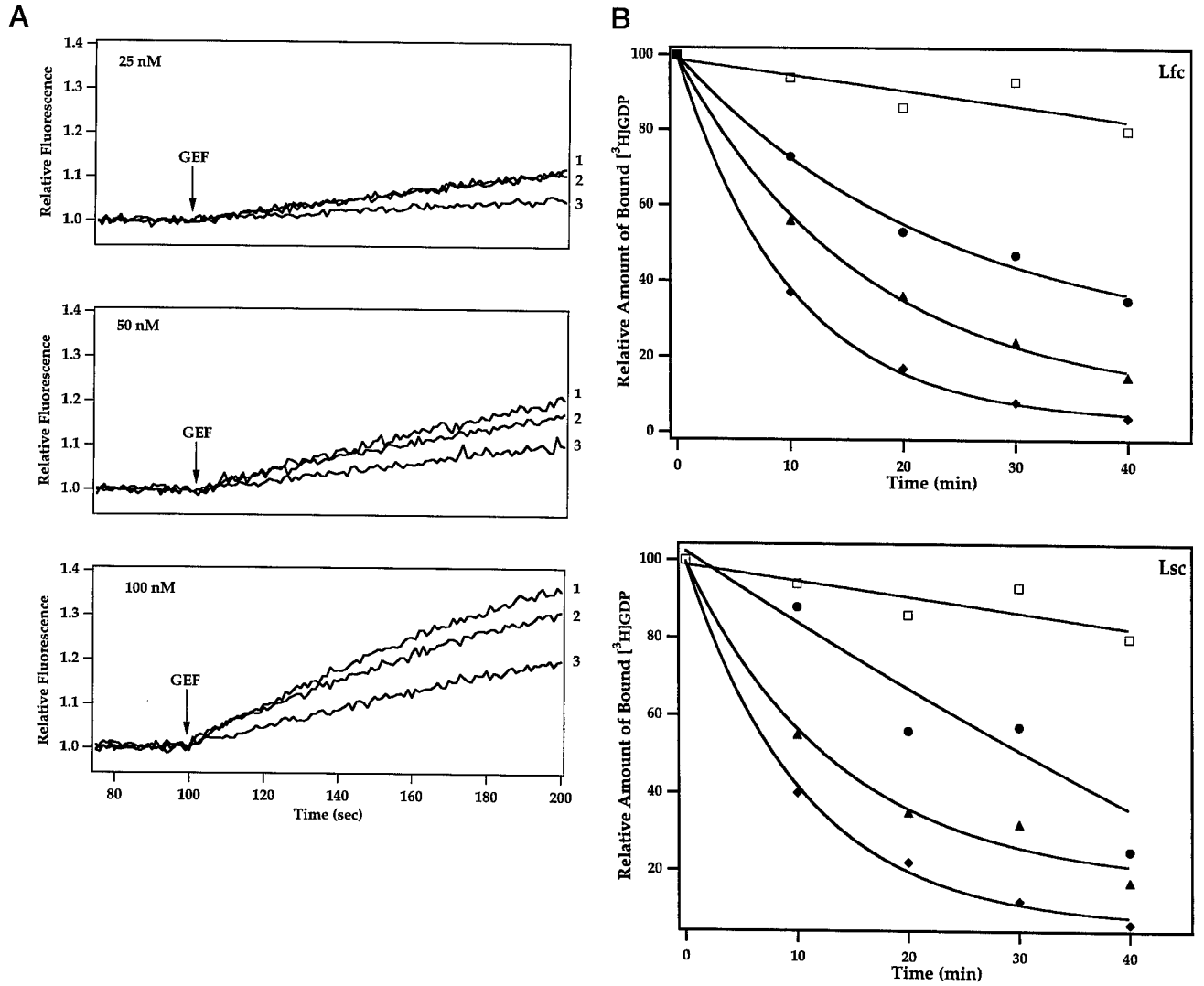


FIG. 4. **Comparison of Lfc, Lsc and Lbc: initial rates of exchange on RhoA and catalytic potential.** *A*, fluorescence spectroscopic analysis of initial rates for guanine nucleotide exchange on RhoA (650 nM) catalyzed by Lbc (*1*), Lfc (*2*), and Lsc (*3*) at 25, 50, and 100 nM. The rate of change of fluorescence was estimated using linear fits to the first 50 s after addition of GEFs. *B*, kinetics of guanine nucleotide exchange catalyzed by Lfc (*top panel*) and Lsc (*bottom panel*), using equimolar concentrations of RhoA and GEF (◆); ▲ represents a 2-fold excess of RhoA to GEF; ● represents a 4-fold excess of RhoA to GEF, and □ represents the GST control.

the exchange event, as monitored by the enhancement of Mant fluorescence. Fig. 4*A* shows that the purified recombinant Lfc, Lsc, and Lbc proteins each stimulated the enhancement of Mant fluorescence as an outcome of catalyzed GDP-Mant-dGTP exchange on RhoA. The time courses for the fluorescence changes stimulated by Lbc and Lfc were virtually identical for each of the three different GEF concentrations assayed. While we consistently found Lsc to stimulate guanine nucleotide exchange at a rate that was ~2-fold slower compared with the rate of exchange stimulated by Lbc and Lfc, it is difficult to know how much significance to attach to these differences because of the difficulties in estimating the protein concentrations for the amount of functional GEF present in the assay.

In order to further compare the catalytic potential of the Lfc and Lsc proteins, we assayed [^{3}H]GDP dissociation from RhoA as catalyzed by different concentrations of these GEFs. We found that guanine nucleotide exchange occurred rapidly when the GEF and GTP-binding proteins were present in equimolar concentrations. As expected, decreasing the concentration of Lfc or Lsc such that RhoA was in 2- or 4-fold excess (over [GEF]) resulted in slower half-times of dissociation. However, it appeared that complete dissociation of [^{3}H]GDP from RhoA will occur at each of the concentrations of Lfc and Lsc assayed, indicating that both of these proteins act catalytically in stimulating the guanine nucleotide exchange reaction.

Specificity of Lfc and Lsc as GEFs—The Lbc protein was shown to be a highly specific GEF for Rho and did not stimulate the guanine nucleotide exchange activity of Cdc42Hs, Rac, or Ras (19). Thus, we examined whether Lfc and Lsc showed similar GEF specificity. The data presented in Fig. 5 indicate that like Lbc, both Lfc and Lsc are highly specific for RhoA when assaying [^{3}H]GDP dissociation after 15 min at room temperature. Neither Lfc nor Lsc showed any GEF activity toward Ras under these conditions, whereas recombinant GST-Ras-GRF strongly stimulated GDP dissociation from Ha-Ras. Similarly neither Lfc nor Lsc showed any activity toward Cdc42Hs, again under conditions where Cdc24 effectively stimulated GDP dissociation. Both Lfc and Lsc were ineffective in stimulating GDP dissociation from Rac. The inability of Lfc and Lsc to serve as GEFs for Ras, Cdc42Hs, and Rac also was observed when complete time courses for [^{3}H]GDP dissociation were obtained (*i.e.* between 2.5 and 30 min (data not shown)). It is interesting that although the DH domains of oncogenic Dbl

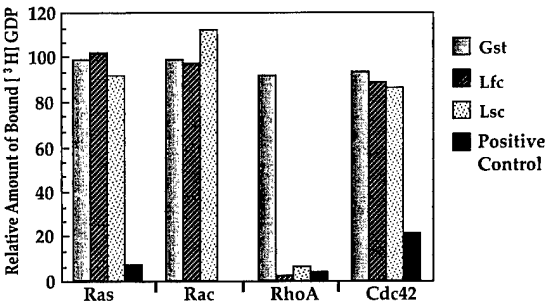


FIG. 5. **Specificity of Lfc and Lsc GEF activity on the Rho and Ras-type GTP-binding proteins.** 2 μg of the various recombinant GTP-binding proteins were preloaded with [^{3}H]GDP and incubated with 2 μg of GST, 1 μg of GST-Lfc, or 1 μg of GST-Lsc before termination of the reaction after 15 min. 1 μg of GST-Ras-GRF was used as a control for assaying stimulated dissociation of GDP from Ras; 1 μg of GST-Lbc was used as a control for stimulated GDP dissociation from RhoA, and 1 μg of Cdc24 was used as a control for stimulated GDP dissociation from Cdc42 and Rac.

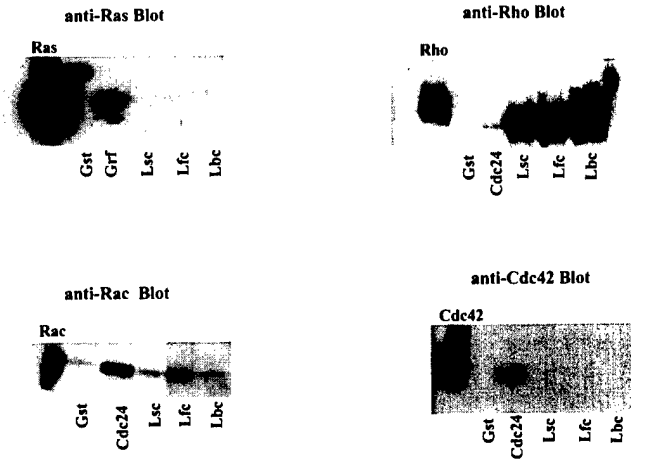


FIG. 6. **Interactions of Lfc and Lsc with the Rho and Ras-type GTP-binding proteins in the nucleotide-free state.** Specific association of Lfc and Lsc with various GTP-binding proteins was determined by using GST-Lfc and GST-Lsc bound to glutathione-agarose beads to precipitate the GTP-binding proteins. The GTP-binding proteins were depleted of nucleotide by preincubation with 10 mM EDTA, as described under "Experimental Procedures." Proteins bound to the beads were resolved by SDS-polyacrylamide gel electrophoresis (12%) and immunoblotted using antibodies directed against the GTP-binding proteins.

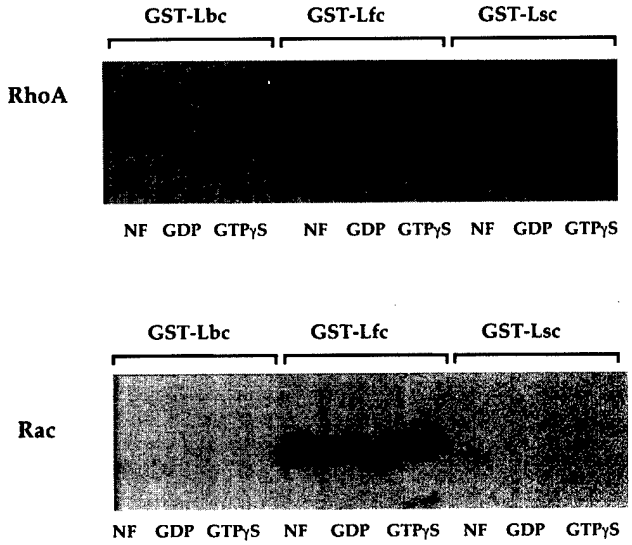


FIG. 7. **Specificity of the nucleotide state of RhoA and Rac for interactions with Lsc and Lfc.** Binding of Lfc, Lsc, and Lbc to RhoA and Rac was determined under different conditions of guanine nucleotide occupancy. Co-precipitation of recombinant RhoA or Rac with GST-Lfc, Lsc, and Lbc fusion proteins was carried out as described in Fig. 6. The nucleotide state of the GTP-binding proteins was established by preincubation in buffer containing 10 mM EDTA (to achieve the nucleotide-depleted state) or buffer containing 10 mM $MgCl_2$ and 200 μM GDP or 200 μM GTPγS for 30 min to establish the GDP-bound and GTPγS-bound states.

(16), Ost (6), and Dbs[2] are able to functionally couple to both Cdc42Hs and RhoA, apparently the related DH domains of Lbc, Lfc, and Lsc are only able to act as GEFs toward Rho proteins. This in turn suggests that the basic framework for GEF specificity toward Rho is contained within the DH domains of the Lbc subfamily of Dbl proteins.

Do Lfc and Lsc Show Similar Binding Specificity for Rho Family Members?—Some members of the Dbl family are able to bind to Rho-related GTP-binding proteins but do not stimulate their guanine nucleotide exchange activity (6, 7). This suggests that in some cases the DH domains serve as binding motifs, perhaps functioning only to recruit GTP-binding proteins. Following this line we wanted to examine whether Lbc, Lfc, and Lsc acted strictly as GEFs, such that they showed similar binding specificity as that exhibited in the GEF assays, or if one or more of these Dbl proteins within the Lbc subfamily were capable of binding to other GTP-binding proteins, in addition to Rho. These experiments were carried out using Lbc, Lfc, and Lsc expressed as GST fusion proteins and immobilized on glutathione-agarose beads. We first assayed the binding specificities of these proteins for different GTP-binding proteins in their guanine nucleotide-depleted state, since this should be the preferred state for binding to GEFs. Fig. 6 shows that as expected, GST-Lbc and GST-Lsc selectively associated with RhoA but not with Rac, Cdc42, or Ras. Also as expected, GST-Ras-GRF formed a complex with Ras and GST-Cdc24 complexed with Cdc42Hs. However, it was surprising that although GST-Lfc bound RhoA as effectively as Lbc and Lsc, the GST-Lfc protein also associated with Rac (as did GST-Cdc24).

We next examined the nucleotide specificity for the binding of RhoA and Rac to the Lbc subfamily members. The results presented in Fig. 7 show that Lbc, Lfc, and Lsc bound specifically to the guanine nucleotide-depleted state of RhoA, again, as is typically the case for GEFs (16). However, the interaction between Lfc and Rac did not demonstrate this specificity, such that Lfc effectively associated with both the GDP- and GTPγS-bound states of Rac as well as with the nucleotide-depleted form of the protein. Neither Lbc nor Lsc showed any binding capability for Rac, regardless of nucleotide state.

Based on the finding that Lfc was able to associate with both the GDP- and GTP-bound forms of Rac, we assayed the ability of Lfc to inhibit GDP dissociation (*i.e.* act as a GDP dissociation inhibitor) or influence GTP hydrolysis (data not shown). All such assays were negative, that is we found no detectable GDP dissociation inhibitor activity for Lfc nor did we find that Lfc could alter the intrinsic or GTPase-activating protein-stimulated GTP hydrolysis of the Rac protein.

DISCUSSION

The Lfc and Lsc oncoproteins are two recently discovered members of the Dbl family of growth regulatory proteins. Each of the members of this family share two motifs, a Dbl homology (DH) domain of ~150 amino acids and a pleckstrin homology (PH) domain that contains ~100 amino acids. Various members of this family including the prototype, Dbl, as well as Cdc24, Tiam-1, and Lbc have been shown to act as guanine

[2] J. A. Glaven, I. P. Whitehead, R. Kay, and R. A. Cerione, unpublished data.

nucleotide exchange factors (GEFs) by stimulating the exchange of GTP for GDP on Rho subfamily GTP-binding proteins (2, 15, 18, 19). In two cases, Cdc24 and Lbc, the GEF activity is highly specific, with Cdc42 serving as the substrate for Cdc24 and Rho serving as the substrate for Lbc. Given the fact that the DH domains of Lfc and Lsc are most similar to that of Lbc, we were interested in the possibility that these two oncoproteins might also act as specific GEFs for Rho and thus together with Lbc comprise a specific subgroup of the larger family of Dbl-related proteins. The data presented here suggest that this in fact is the case. Both Lfc and Lsc appear to be highly specific GEFs for Rho and show no detectable GEF activity toward Cdc42, Rac, or Ras. The abilities of these oncoproteins to stimulate GDP dissociation from or GDP-[^{35}S]GTPγS exchange on Rho are similar to the activities measured for Lbc, both with regard to the initial rate of GDP dissociation and the catalytic capability of their GEF activities. These findings then suggest that the essential features for GEF specificity for Rho are contained within the DH domains of Lbc, Lfc, and Lsc but are missing in the Dbl and Ost oncoproteins, since the latter two proteins functionally couple to Cdc42 as well as to Rho.

An obviously important question that is raised by these studies concerns the reason for the existence of multiple GEFs for Rho. One possibility might have been differences in tissue distribution; for example, if Lbc, Lfc, and Lsc showed markedly different tissue locations, then the need for multiple Rho GEFs would be obvious because of the ubiquitous distribution of Rho. However, the fact that all three of these oncoproteins appear to be located in similar tissues, and in the case of Lfc and Lsc, the same cell types, seems to argue against this explanation.

A second possibility may involve distinct cellular locations. There are already indications that one Rho subfamily member, Cdc42, is located both in the plasma membrane and in Golgi membranes (34) and that it may be necessary to activate Cdc42 at both of these cellular locations. The targeting of Dbl and Dbl-related proteins to specific cellular sites through their PH domains may provide a means to selectively activate Rho subfamily proteins at distinct locations. In the case of Dbl, the PH domain appears to target this GEF to a cytoskeletal location (17), whereas in the case of Lfc, the PH domain appears to be targeting the GEF to the plasma membrane, since replacement of the PH domain with a Ras-farnesylation sequence restores transforming activity to Lfc (13). The cellular locations of Lbc and Lsc have not yet been determined, although chances are that their PH domains will bind specific cellular targets. Thus, it will be interesting in the future to determine whether Lsc and Lbc are located in different regions of the cell relative to Lfc.

A third rather intriguing possibility for the existence of what appears to be multiple Rho GEFs concerns the potential involvement of these proteins in different signaling pathways mediated by other GTP-binding proteins (*i.e.* aside from Rho). This directly bears on the question of whether all proteins that contain DH domains act directly as GEFs or, if in some cases, these proteins serve to recruit GTP-binding proteins to a specific cellular site (as marked by the PH domain) where they can either be activated or regulated by other factors (*e.g.* lipids). There already is precedent for Dbl-related proteins binding GTP-binding proteins without having direct effects on guanine nucleotide exchange. One such example is Ect2 (7), which binds Rac in a guanine nucleotide-independent manner, and a second example is Ost (6), which associates specifically with GTP-bound Rac. In these studies, we show that Lfc, unlike Lbc or Lsc, binds to Rac in a guanine nucleotide-independent manner. This interaction does not appear to have any direct influence (either stimulatory or inhibitory) on GDP dissociation from Rac nor on GTP hydrolysis. However, it is interesting that recent work has shown that expression of Lfc (but not Lsc) in COS cells results in an activation of the c-Jun kinase (JNK1),[3] a nuclear mitogen-activated protein kinase that catalyzes the amino-terminal phosphorylation of c-Jun. Given that it has been well documented that Rac and Cdc42, but not Rho, initiate signaling cascades that culminate in the activation of JNK1 (24–27), it will be interesting to see if the Lfc-Rac interaction reported here is in some way involved in the pathway that mediates Lfc effects on JNK1 activity.

An important direction of future studies will be to further investigate the functional outcome of Lfc-Rac interactions. Recently, we have found that phosphatidylinositol 4,5-bisphosphate may represent an alternative factor for initiating the activation of Rho subtype GTP-binding proteins (35). Specifically, it appears that phosphatidylinositol 4,5-bisphosphate can bind directly to Cdc42 (and to lesser extents to Rac and Rho) and strongly stimulate GDP dissociation. While it does not appear that phosphatidylinositol 4,5-bisphosphate will act cooperatively with Dbl (since Dbl, alone, is fully capable of stimulating GDP-GTP exchange on Cdc42 or Rho), it will be interesting to see if phosphatidylinositol 4,5-bisphosphate or other lipids can act synergistically with Lfc to influence the activation state of Rac. The location of a potential lipid binding domain on Lfc raises other possibilities regarding lipid factors that might influence the functional coupling of this oncoprotein to Rho-like GTP-binding proteins. Thus, it may be that the family of Dbl-related proteins provide a broader group of functions than originally anticipated, not only acting directly as specific GEFs but also specifying the cellular sites where other factors can promote the activation of GTP-binding proteins and/or where targets can bind to GTP-binding proteins and mediate downstream signaling events.

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Identification of the Eukaryotic Initiation Factor 5A as a Retinoic Acid-stimulated Cellular Binding Partner for Tissue Transglutaminase II*

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GTP-binding protein/transglutaminases (tissue transglutaminases or TGases) have been implicated in a variety of cellular processes including retinoic acid (RA)-induced apoptosis. Recently, we have shown that RA activates TGases as reflected by stimulated GTP binding, increased membrane association, and stimulated phosphoinositide lipid turnover. This prompted us to search for cellular proteins that bind TGases in a RA-stimulated manner. In this report, we show that the eukaryotic initiation factor (eIF-5A), a protein that is essential for cell viability, perhaps through effects on protein synthesis and/or RNA export, associates with the TGase *in vivo*. The interaction between eIF-5A and TGase is specific for the GDP-bound form of the TGase and is not detected when the TGase is pre-loaded with GTPγS. The TGase-eIF-5A interaction also is promoted by Ca^{2+}, Mg^{2+}, and RA treatment of HeLa cells. In the presence of retinoic acid, millimolar levels of Ca^{2+} are no longer required for the TGase-eIF-5A interaction. Nocodazole treatment, which blocks the cell cycle at mitosis (M phase), strongly inhibits the interaction between eIF-5A and cytosolic TGase. The interaction between TGase and eIF-5A and its sensitivity to the nucleotide-occupied state of the TGase provides a potentially interesting connection between RA signaling and protein synthesis and/or RNA trafficking activities.

Tissue transglutaminases (TGases)[1] represent an interesting class of enzymes that were originally identified based on their ability to catalyze the Ca^{2+}-dependent formation of covalent bonds between peptide-bound glutamyl residues and the primary amino groups in a variety of compounds (1). It was then realized that these enzymes are regulated by GTP and in fact can undergo a GTP-binding/GTPase cycle like classical G proteins (2–4). More recently, it also has been appreciated that these enzymes are capable of ATP hydrolysis at a site distinct from the GTP-binding site (5). The TGases appear to be widely distributed within the cell, mainly present in the cytosol (6, 7), but are also found in plasma membranes (8) and in the nucleus (9). They have been implicated in a variety of fundamentally important cellular functions, including cell adhesion, wound healing, and cellular differentiation and apoptosis (10–13). It seems likely that the TGases play a role as signaling transducers in some of these biological responses (again reminiscent of classical G proteins), with one particular example being the mediation of α1-adrenergic receptor regulation of phosphoinositide lipid turnover through the ability of the GTP-bound TGase to stimulate phospholipase C-δ1 (14).

Our laboratory has been interested in further probing the possible importance of the GTP-binding/GTPase cycle of TGases in their biological function. Recently, we found that retinoic acid (RA) treatment of HeLa cells under conditions that give rise to cellular differentiation and apoptosis strongly stimulated the GTP binding activity of the TGases (15). This was accompanied by an increased association of TGase with the plasma membrane and a concomitant stimulation of phospholipase C activity. These RA-stimulated effects could not be attributed to changes in the expression of the TGase, which caused us to propose that exposure of cells to RA may result in the expression of specific regulatory factors for the TGase, *e.g.* lipid-modifying enzymes and/or other proteins capable of imparting posttranslational modifications such as protein kinases.

In the present study, we set out to extend this work and identify possible regulatory factors as well as other potential target/effector molecules for the TGase. In particular, we followed up on an observation that an ~18-kDa protein appeared to be co-purifying with TGase from rabbit liver preparations. We have identified this protein as the eukaryotic initiation factor (eIF-5A), which was originally suspected to play an important role in protein synthesis but more recently has also been suggested to participate in nuclear (RNA) export (16). We show here that eIF-5A is a specific cellular binding partner for a particular form of the TGase and that this interaction is stimulated by RA, thus suggesting an interesting and potential link between protein synthesis and/or RNA trafficking and RA regulation of cellular activities.

EXPERIMENTAL PROCEDURES

Construction of a Recombinant Baculovirus to Express Human Transglutaminase in Spodoptera frugiperda Sf21 Cells—The cDNA clone of human tissue TGase was kindly provided by Dr. Peter Davies (University of Texas Medical School, Houston) in a pSG5 vector (Stratagene). It was digested with *Nco*I, and the resultant 2.2-kilobase fragment, including the coding region of the protein, was ligated into pGEXKG-lin (17), which was predigested with *Nco*I so that the TGase gene was fused in-frame downstream of the glutathione *S*-transferase (GST) coding region of the vector. The resultant construct was designated pGEXKG-lin-TG. The *Xba*I fragment of the pGEXKG-lin-TG containing the GST-tagged TGase was further subcloned into the baculovirus expression vector pVL1393 at the *Xba*I site.

The baculovirus expression vector containing GST-TGase was co-transfected with BaculoGold DNA into Sf21 cells using the BaculoGold transfection kit (Pharmingen, San Diego, CA). Recombinant viruses were isolated by plaque purification (18). Sf21 cells were infected by recombinant viruses at a multiplicity of infection of 5 plaque forming

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[1] The abbreviations used are: TGases, transglutaminases; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; RA, retinoic acid; eIF-5A, eukaryotic initiation factor-5A; GST, glutathione *S*-transferase; DTT, dithiothreitol; TTBS, Tris-buffered saline, 0.2% Tween 20; PAGE, polyacrylamide gel electrophoresis.

units/cell. The infected cells were harvested 60 h postinfection, and the cell pellets were stored frozen at −80 °C.

Protein Purification of TGase from Sf21 Cells—The pellets were thawed (50% w/v) in buffer A (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 100 μM phenylmethylsulfonyl fluoride and 10 μg/mL of leupeptin and aprotinin each) containing 1% Nonidet P-40 and incubated at 4 °C for 1 h. The suspension was centrifuged at 12,000 × *g* for 1 h, and the supernatant was mixed with glutathione-agarose beads (10% v/v) and incubated at 4 °C by continuous shaking for 1 h. The suspension was then centrifuged at 1000 × *g* for 10 min, and the beads were washed three times with buffer A and suspended in the same buffer (50% w/v) for storage.

Cell Culture—HeLa cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml of penicillin and streptomycin, 2.0 g/liter sodium bicarbonate, and 10 mM HEPES in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C. To achieve cell-cycle arrest in M phase, nocodazole (80 ng/ml) was added to the growth medium, and cells were allowed to grow for 24 h. For harvesting, the cells were trypsinized and washed with RPMI 1640 containing 10% fetal calf serum.

Cell Fractionation—Cells were pelleted and washed twice in ice-cold hypotonic buffer containing 10 mM HEPES, pH 8.0, 5 mM KCl, and 2 mM $MgCl_2$. The cell pellets were then quick frozen in liquid nitrogen for storage. Thawed cells were resuspended in buffer B (25 mM HEPES, pH 7.4, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, and 10 μg/ml aprotinin and leupeptin each). The suspension was homogenized using a Potter-Elvehjem homogenizer. Cellular debris was spun out at 3000 × *g* for 15 min. The supernatant was collected and spun at 100,000 × *g* for 45 min. Nonidet P-40 was added to the supernatant to a final concentration of 1% (this was used as a cytosolic fraction), and the membrane pellet was resuspended in 1% Nonidet P-40 in 25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM benzamidine, 100 μM phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin and leupeptin each.

Immunoprecipitations and Precipitation with Glutathione-Agarose—For the precipitation of eIF-5A by the GST-TGase fusion protein, the clear supernatant representing the cytosolic fraction of HeLa cells was transferred to fresh microcentrifuge tubes. It was mixed with a suspension (25 μl) of GST-TGase beads and incubated for 2 h at 4 °C in the presence of different concentrations of salts ($CaCl_2$ and $MgCl_2$) and nucleotides (GDP and GTPγS) as indicated in the figures. The suspension was centrifuged, and the pellet was washed three times with buffer A and finally suspended in two times Laemmli sample buffer (19) and boiled for 5 min. The supernatant was used for the purpose of electrophoresis and Western blotting.

For the immunoprecipitation of eIF-5A, supernatants (200 μl) were incubated for 1 h at 4 °C with 5 μl of rabbit anti-eIF-5A polyclonal antibody, kindly provided by Dr. Edith Wolff (NIH, Bethesda, MD). Protein A-Sepharose (5 mg/ml lysate) was then added to the sample and incubated for 1 h at 4 °C. A parallel set of control samples containing the same amount of rabbit IgG was also performed. The immunoprecipitates were pelleted and washed three times with buffer 1 (137 mM NaCl, 16 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4, containing 1% Nonidet P-40) and resuspended (50% v/v) in buffer A containing 1% Triton X-100. All of the TGase-eIF-5A co-precipitation data shown under "Results and Discussion" were representative of three experiments.

Identification of Proteins by Western Blot Analyses—20 μl of the immunoprecipitates were diluted with an equal volume of 2 × Laemmli sample buffer (19) and boiled for 5 min. The supernatants were subjected to electrophoresis on a 10% polyacrylamide gel with a 4% stacking gel. Proteins were transferred to 0.45 μM polyvinylidene fluoride filters (Millipore Corp.), and the filters were blocked using Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.4)/2% bovine serum albumin for 1 h. The blots were exposed to primary antibody against TGase in Tris-buffered saline, 0.2% Tween 20 (TTBS) or against eIF-5A in TTBS at a 1:250 dilution or a 1:500 dilution, respectively, and were washed three times at 15 min intervals. The blots were then exposed to secondary antibody (anti-mouse horseradish peroxidase or anti-rabbit horseradish peroxidase, Amersham Corp.) at a 1:5000 dilution in TTBS, 1% bovine serum albumin for 1 h, then washed three times with TTBS and two times with Tris-buffered saline, and visualized using a Chemiluminescence system (ECL, Amersham Corp.).

Photoaffinity Labeling of GTP-binding Proteins—Photoaffinity labeling of GTP-binding proteins with [α-^{32}P]GTP was carried out as described previously (9). Samples (20 μl) were incubated with 5 mM EDTA and 2–3 μCi [α-^{32}P]GTP (0.02 μM) (20 μl final volume) in tissue culture plates (96 wells) for 10 min at room temperature in a reaction buffer that contained 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM AMP(PNP). The samples were then placed in an ice bath, irradiated with UV light (254 nm) for 15 min, mixed with 5 × sample buffer (19), and boiled for 5 min. SDS-PAGE was performed (10% polyacrylamide), and then the gels were stained, dried, and exposed on Kodak X-OMAT XAR-5 film using a DuPont image intensifier screen.

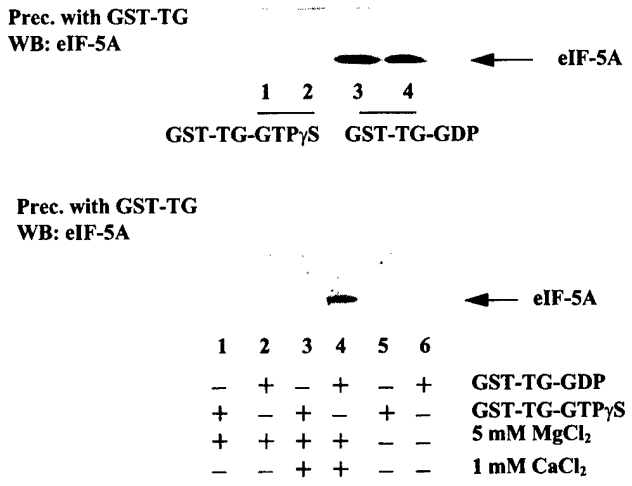

FIG. 1. **Nucleotide dependence of the association between eIF-5A and TGase.** The insect cell-expressed GST-TGase (10 μg on glutathione beads) was resuspended in 100 μl of 50 mM HEPES-NaOH, pH 7.4, 1 mM DTT, 100 mM NaCl, and 1% Nonidet P-40 and was mixed with 100 μl of a HeLa cell cytosolic fraction that was precleared with GST beads. The suspension was incubated with either 100 μM GDP or 100 μM GTPγS in the presence of 1 mM $CaCl_2$ and 5 mM $MgCl_2$ (*top panel*) or as indicated in the *bottom panel*. After incubating at 4 °C for 1 h, the suspension was centrifuged, and the beads were washed three times with the washing buffer A containing 1% Nonidet P-40 as indicated under "Experimental Procedures." The pellet was mixed with the sample buffer, run on SDS-PAGE, and transferred to nitrocellulose paper. The paper was Western blotted using an eIF-5A polyclonal antibody. The protein bands were visualized using the ECL (Amersham) system. Shown in the figure is the band for eIF-5A. Various other nonspecific bands are often observed with apparent mobilities ranging from 30 to 100 kDa (not shown).

RESULTS AND DISCUSSION

Various lines of evidence have suggested that GTP-binding protein/transglutaminases (TGases) may represent a new family of transducer molecules that upon binding GTP can stimulate target/effector activities such as phosphoinositide lipid turnover (8, 20). We recently reported that a number of biochemical activities of TGases are stimulated by RA including GTP binding (and the resultant GTP hydrolytic activity), transglutaminase activity, plasma membrane-association and phosphoinositide lipid turnover (15). The regulatory mechanisms underlying these RA-stimulated activities are not known, although they cannot be attributed to simple changes in the levels of TGase expression (15). As a means to gain further insight into the interplay between RA and TGases, as well as into the function of TGases, we have set out to identify potential regulators and other binding partners or targets for these GTP-binding proteins.

Based on an earlier observation that an ~18-kDa protein appeared to co-purify with TGases from rabbit liver,[2] we obtained sufficient amounts of the 18-kDa protein for microsequence analysis and determined that it was the eukaryotic initiation factor-5A (eIF-5A). At present, little is known regarding the cellular functions of eIF-5A. It has been suggested to participate in some aspect of protein synthesis at the ribosomal subunit-joining step and/or at a later stage of 80 S ribosomal

[2] U. S. Singh, unpublished results.

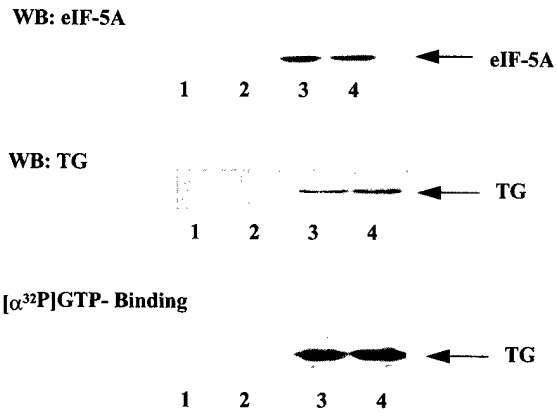

FIG. 2. **Immunoprecipitation of TGase with a polyclonal antibody against eIF-5A.** An aliquot (100 μl) of the cytosolic fraction was immunoprecipitated with a polyclonal antibody (eIF-5A-S) raised against human eIF-5A (*lanes 3* and *4* are duplicates) or with a control antibody (rabbit IgG) (*lanes 1* and *2* are duplicates) as outlined under "Experimental Procedures." The proteins were electrophoresed on SDS-PAGE, transferred to nitrocellulose, probed with the eIF-5A antibody, and visualized with ECL (*top panel*), or the immunoprecipitated proteins were first labeled with [α-^{32}P]GTP, electrophoresed, and visualized by autoradiography (*bottom panel*). The blot was then stripped (50 mM Tris-HCl, pH 8.0, 0.1% SDS, and 50 mM β-mercaptoethanol) for 2 h at 70 °C, reprobed with monoclonal antibody (CUB 7402) raised against guinea pig liver transglutaminase, and visualized with ECL (Amersham) (*middle panel*). In these experiments, the autorads and blots were exposed overnight.

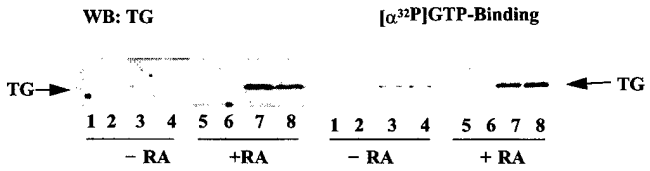

FIG. 3. **Effect of retinoic acid on the association between eIF-5A and TGase.** The cytosolic fraction (100 μl) isolated from the RA-treated and -untreated HeLa cells was immunoprecipitated with eIF-5A antibody or control antibody as indicated under "Experimental Procedures." The immunoprecipitates were suspended in the reaction buffer for photoaffinity labeling and used for GTP binding by UV irradiation. The proteins were electrophoresed on SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography (*right panel*) or by Western blotting with the anti-TGase antibody (*left panel*). In these experiments, the autorads and blots were exposed for 2 min. *Lanes 1, 2, 5,* and *6* represent immunoprecipitates using control antibody incubated with the cytosolic fractions prepared from the control and RA-treated HeLa cells. *Lanes 3, 4, 7,* and *8* represent the immunoprecipitates using eIF-5A antibody incubated with control and RA-treated HeLa cell cytosolic fractions.

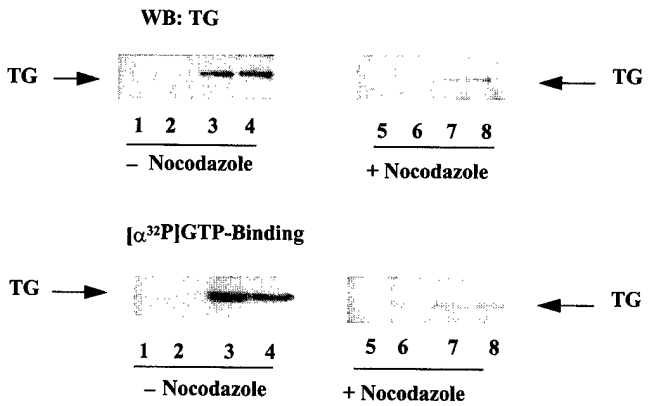

FIG. 4. **Effects of nocodazole on the association between eIF-5A and TGase.** Immunoprecipitation with an anti-eIF-5A polyclonal antibody (*lanes 3* and *4*) or IgG control antibody (*lanes 1* and *2*) was performed by using aliquots (100 μl) of the cytosolic fractions from control and nocodazole-treated HeLa cells as described under "Experimental Procedures" and in the legend to Fig. 2. The proteins were electrophoresed on SDS-PAGE, transferred to nitrocellulose, probed with the TGase monoclonal antibody, and visualized by ECL (*top panel*) or were photolabeled with [α-^{32}P]GTP (*bottom panel*).

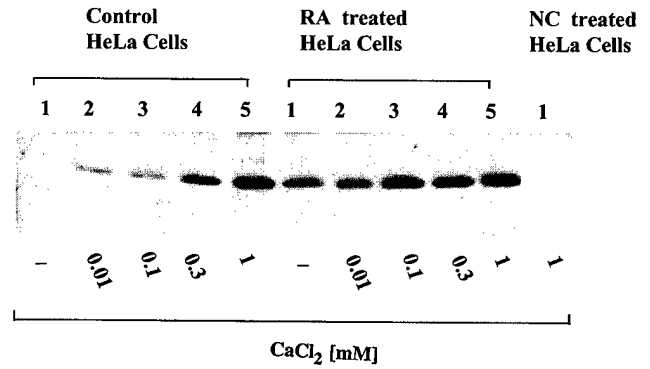

FIG. 5. **Effects of retinoic acid, Ca^{2+}, and nocodazole on the interactions between eIF-5A and GST-TGase.** The binding of eIF-5A to GST-TGase was assayed in control HeLa cells, RA-treated HeLa cells, and nocodazole-treated HeLa cells as described in the legend to Fig. 1 and at the indicated levels of $CaCl_2$.

initiation complex formation (21). Recently, eIF-5A also has been suggested to play a role in RNA export, based on its interaction with the HIV-1 protein, Rev (16). The eIF-5A protein is unique in that it is the only cellular protein known to contain the unusual amino acid hypusine; a number of lines of evidence indicate that hypusine and eIF-5A are essential for eukaryotic cell viability (22). Likewise, there are a number of indications for an important involvement of TGases in cell cycle progression and cell growth regulation (6, 13, 23–26). Thus, a TGase-eIF-5A interaction would have interesting implications, particularly regarding the possible connection of RNA metabolism to growth regulation (27).

We first set out to determine whether we could obtain biochemical evidence that eIF-5A is in fact a binding partner for TGase. To do this, we expressed the TGase in insect cells as a GST fusion protein to use this as an affinity reagent for identifying eIF-5A as a possible target. The results presented in Fig. 1 (*top panel*) show that when the GST-TGase protein was incubated with lysates from HeLa cells, the endogenous eIF-5A (as detected by Western blotting with an anti-eIF-5A antibody) was co-precipitated with GST-TGase upon the addition of glutathione-agarose. This interaction appeared to be specific for the GDP-bound form of the GTP-binding protein, since we could not detect an interaction with eIF-5A when the GST-TGase was preloaded with GTPγS. The GST-TGase-eIF-5A interaction also required both Ca^{2+} and Mg^{2+} (Fig. 1, *lower panel*; also see below).

We next examined whether the TGase-eIF-5A interaction occurred in intact cells. Using an anti-eIF-5A polyclonal antibody that can immunoprecipitate eIF-5A from cells (Fig. 2, *top panel*), we found that TGase was specifically co-precipitated with eIF-5A (Fig. 2, *middle panel, lanes 3* and *4*) but not with nonimmune IgG (Fig. 2, *middle panel, lanes 1* and *2*) as indicated by Western blotting with an anti-TGase antibody. The TGase that co-precipitates with eIF-5A can also be photoaffinity labeled with [α-^{32}P]GTP (Fig. 2, *lower panel, lanes 3* and *4*).

The ability of the eIF-5A-associated TGase to bind GTP indicates that it belongs to an activated pool of the endogenous TGase. Previously, we had shown that in HeLa cells most of the TGase was inactive and not capable of binding [α-^{32}P]GTP (15). However, as alluded to earlier, RA, which causes the cells to differentiate and undergo apoptosis (13), strongly stimulated the GTP binding activity of the cellular TGase (15). Since this

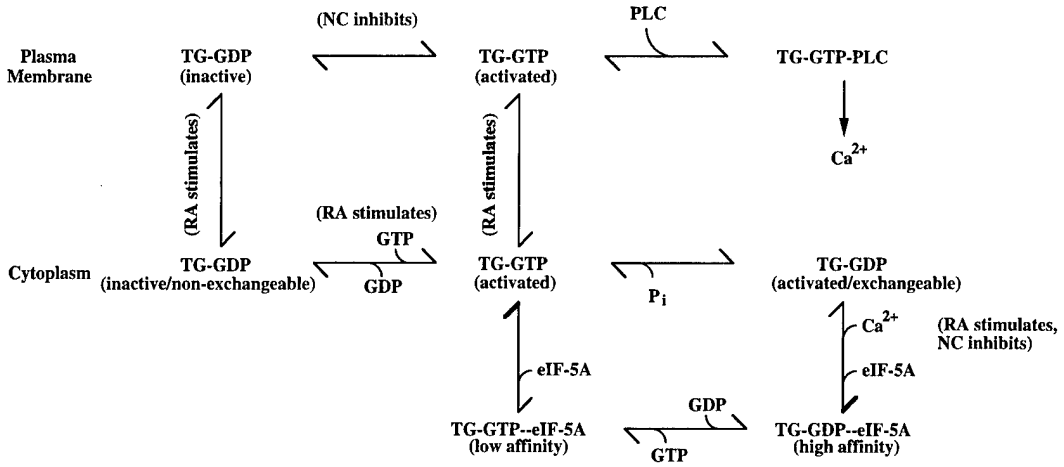

FIG. 6. **Schematic representation of the interaction between eIF-5A and TGase.** Previous studies have shown that RA treatment of cells results in the conversion of the cytosolic GTP-binding protein/transglutaminase (*TG*) pool from an inactive fraction to an activated fraction (*i.e.* a pool of TG capable of undergoing GTP-GDP exchange and/or binding GTP). RA treatment also increases the association of TG with the plasma membranes. Normally this is the activated pool of TG but in nocodazole (*NC*)-treated cells, the membrane-associated TG is inactive (incapable of GTP-GDP exchange). The activated membrane-associated pool of TG elicits a GTP-dependent stimulation of phospholipase C activity, giving rise to an increase in intracellular Ca^{2+}. This Ca^{2+} signal may increase the binding affinity of the GDP-bound (activated/nucleotide exchangeable) cytosolic pool of TG for eIF-5A. RA treatment reduces the requirement for millimolar levels of Ca^{2+} for the TG-eIF-5A interaction, whereas NC treatment inhibits this interaction even in the presence of millimolar Ca^{2+}. It is further proposed that GTP binding weakens the binding affinity of TG for eIF-5A.

did not appear to be due to a change in the expression of the TGase, we proposed that RA treatment of cells increased the expression of a lipid-modifying enzyme and/or possibly another regulatory factor (*e.g.* a protein kinase) that modified the TGase, thus creating an activated pool of protein that was capable of GTP binding activity and an increased ability to associate with the plasma membrane. Given this suggestion, we therefore reasoned that if it was the activated pool of TGase that associates with eIF-5A, then RA treatment of cells should stimulate the formation of a TGase·eIF-5A complex. The results presented in Fig. 3 show that this in fact is the case. Specifically, following RA treatment of HeLa cells, TGase was detected in co-immunoprecipitates with eIF-5A even after only a very brief exposure (~2 min) of the blot and under conditions where in the absence of RA, little detectable TGase was present in the eIF-5A precipitates. Fig. 3 (*right panel*) also shows that RA treatment strongly increased the amount of [α-^{32}P]GTP binding activity (corresponding to the TGase) that co-immunoprecipitated with eIF-5A.

We have been able to further probe the connection between RA-promoted activation of TGase and its interaction with eIF-5A by taking advantage of a recent finding that nocodazole treatment of HeLa cells (under conditions that cause the cells to accumulate in M phase) (28) inhibits the ability of membrane-associated TGase to bind GTP and thus uncouples the ability of the RA-regulated TGase to associate with plasma membranes from its ability to become activated (data not shown). It is interesting that nocodazole treatment also strongly inhibits the association of the cytosolic TGase with eIF-5A. The results presented in Fig. 4 clearly show that the co-precipitation of TGase with eIF-5A does not occur in HeLa cells that have been pretreated with nocodazole, either when monitoring this interaction by Western blotting with the specific anti-TGase antibody (*upper panel*) or by photoaffinity labeling with [α-^{32}P]GTP (*lower panel*). A similar inhibitory effect by nocodazole was also obtained with RA-treated HeLa cells (data not shown)

The results presented in Fig. 5 show that the effects of both RA and nocodazole treatment are directed at eIF-5A as well as at the membrane-bound pool of TGase. Given that Ca^{2+} appeared to serve as a necessary cofactor for TGase-eIF-5A interactions (see Fig. 1), we performed an experiment to determine whether treatment with RA or nocodazole influenced the ability of Ca^{2+} to stimulate these interactions. Specifically, lysates were prepared from control HeLa cells and from RA-treated cells or nocodazole-treated cells and then incubated with the recombinant GST-TGase in the presence of different levels of $CaCl_2$. The GST-TGase·eIF-5A complexes were precipitated with glutathione-agarose and Western blotted with the anti-eIF-5A polyclonal antibody. As shown in Fig. 5, the GST-TGase-eIF-5A interaction was maximal when lysates from control cells were incubated with 1 mM $CaCl_2$, whereas in RA-treated cells GST-TGase-eIF-5A interactions were clearly detected even in the absence of added $CaCl_2$. These results suggest that RA treatment has a direct effect on eIF-5A, such that it is able to associate with the TGase even at low (micromolar) levels of Ca^{2+}. It should be noted that nocodazole treatment completely eliminated the interaction between GST-TGase and eIF-5A. Because nocodazole did not decrease the levels of eIF-5A detected in these lysates by Western blotting (data not shown), these findings suggest that the nocodazole effects on TGase-eIF-5A interactions are directed at eIF-5A, such that it cannot bind to the TGase even in the presence of millimolar levels of Ca^{2+}. Thus, like RA, nocodazole exhibits regulatory effects on both partners of the TGase-eIF-5A binding interaction.

These results when taken together with previous findings are consistent with the following model (Fig. 6). Treatment of HeLa cells with RA promotes the formation of an activated pool of the TGase, which has an increased affinity for the plasma membrane and the capability to undergo GTP/GDP exchange. That portion of the membrane-associated TGase that binds GTP can then stimulate the activity of a phospholipase C enzyme (15); recent work would suggest that this is the phospholipase-δ1 isoform (14). The TGase-promoted stimulation of phospholipase C activity gives rise to an increase in intracellular Ca^{2+} which has been suggested to stimulate the enzymatic tranglutaminase activity of the TGase (29). If Ca^{2+} binding to the TGase has some type of regulatory affect on the tranglutaminase active site, such an effect might account for the ability of Ca^{2+} to promote TGase-eIF-5A interactions. The involvement of the transglutaminase active site in binding

eIF-5A also might explain an earlier observation that the unique hypusine residue of eIF-5A serves as substrate for transglutaminase reactions, *i.e.* it can be cross-linked to the γ-carboxamide group of glutamine side chains (30). Within this scheme, RA could promote TGase-eIF-5A interactions both by initiating a cascade of events that results in the membrane association of TGase and a resultant stimulation of phospholipase C activity and increase in intracellular Ca^{2+}, and by having an as yet undetermined effect on the eIF-5A molecule (perhaps by influencing some type of posttranslational modification), such that eIF-5A can bind the TGase at micromolar levels of Ca^{2+}. Nocodazole treatment by inhibiting the activation of membrane-associated TGase, as well as by exerting a regulatory effect on eIF-5A, inhibits TGase-eIF-5A interactions.

Clearly, a critical question concerns how the TGase-eIF-5A interaction influences the normal functions of these proteins. We have not found any affect on the various measurable TGase activities; however, as alluded to earlier, an interesting possibility is that TGases influence the cellular localization of eIF-5A. When the TGase is in a GDP-bound state (following GTP hydrolysis (31)), it is able to bind eIF-5A and maintain eIF-5A in the cytoplasm. However, upon GTP-GDP exchange, the eIF-5A is released perhaps allowing the molecule to bind to some other cytosolic (target) protein involved in protein synthesis and/or to return to the nucleus to participate in some aspect of RNA trafficking. Thus, the GTP-binding/GTPase cycle of the TGase could serve as a timing device to regulate changes in the cellular localization of eIF-5A that are important for either protein synthesis or RNA trafficking between the cytoplasm and the nucleus. The fact that nocodazole treatment perturbs both the GTP-binding/GTPase cycle of membrane-associated TGase, and the ability of eIF-5A to associate with the TGase, further suggests that eIF-5A activity might be coupled to the cell cycle. Taken together, these results would suggest that other factors that influence cell-cycle progression may also exert regulatory effects on TGase-eIF-5A interactions. Future studies will be directed toward testing the different possibilities raised above and determining the identity of additional cellular factors that mediate the regulation of TGase activities by RA as well as additional binding partners for eIF-5A.

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The Nuclear Cap-Binding Complex is a Novel Target of Growth Factor Receptor-Coupled Signal Transduction

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ABSTRACT

In an attempt to further understand how nuclear events (such as gene expression, nuclear import/export, and cell cycle checkpoint control) might be subject to regulation by extracellular stimuli, we sought to identify nuclear activities which were sensitive to the addition of growth factor to serum-arrested cells. Using a photoaffinity labeling approach, we detected an 18 kDa, nuclear protein (originally designated p18) which responded to growth factor treatment of cells with an increased ability to bind GTP. This GTP-binding response was also observed when cells were arrested in the G_1/S phase of the cell cycle and when cells were stressed with UV irradiation, suggesting that p18 may function downstream of multiple cellular signaling systems. Gel filtration analysis suggested that p18 lacked GTP-binding activity when existing in a monomeric state but required the formation of a higher molecular weight complex in order to bind GTP. Through a combination of approaches, including purification, immunoprecipitation and Western blotting, and nucleotide competition experiments, we determined that p18 is identical to the RNA ^{7}methylguanosine cap-binding protein, CBP20. CBP20, together with an 80 kDa protein, CBP80, forms the nuclear cap-binding complex (CBC) which has been implicated in RNA metabolism at the level of pre-messenger RNA splicing and RNA export from the nucleus. We show here that the CBC binds to capped RNA in a growth factor-dependent manner. Taken together, these data identify the CBC as a new nuclear target for growth factor-coupled signal transduction and suggest novel mechanisms by which growth factors can influence gene expression and cell growth.

INTRODUCTION

Growth factor binding to cell surface receptors can initiate signals which are propagated through the cell by a cascade of protein-protein interactions, ultimately to impact upon specific cellular functions and regulate cell growth. The activities of signaling molecules must be tightly regulated in order to maintain the integrity of cellular communication, as loss of regulation in these processes can give rise to defects in cell growth and metabolism that may lead to human disease. The low molecular weight GTP-binding protein, Ras, provides a classical example of how the loss of coordinated regulation of a signaling molecule can lead to aberrant cell growth. The GTP-binding/GTPase activity of Ras provides the cell with a tightly regulated molecular switching mechanism, such that Ras is inactive in its GDP-bound conformation, but upon growth factor-stimulated GTP-binding, is able to interact with downstream target or effector molecules and thereby propagate mitogenic signals to the nucleus (1). Mutations in Ras that disrupt its switching activity (i.e. GTPase-defective mutations which lock Ras into its GTP-bound, active conformation) are found in approximately 40% of human cancers (2).

Given the importance of these signaling processes in cell growth, a great deal of effort has gone into the elucidation of proteins participating in signaling pathways which start at the level of receptor activation and culminate in the stimulation of a nuclear activity. Multiple cascades have now been identified that result in the activation of different nuclear MAP kinases including the ERKs (extracellular receptor-activated kinase) and the stress-responsive JNK/SAPK (Jun N-terminal kinase/ stress-activated protein kinase) and p38 (1, 3). ERK activation is the outcome of mitogen-stimulated Ras-signaling, whereas JNK/SAPK and p38 activities are stimulated by pathways involving the Cdc42 and Rac GTP-binding proteins (3-7). While these different signaling pathways were originally thought to be independently regulated, later work showed that cross-talk between the individual MAP kinase pathways exists. A common functional outcome of

the activation of these signaling pathways is a translocation of the activated MAP kinase to the nucleus and subsequent activation of specific transcription factors and gene expression (3-7).

How other nuclear functions might be influenced in response to extracellular stimulation is less clear, although it is attractive to envision how critical nuclear activities such as RNA metabolism and export, nuclear protein import, and cell cycle control might be subject to regulation as downstream targets of extracellular stimuli. With this in mind, we set out to identify novel nuclear activities which were growth factor-responsive. In particular, we were interested in the possibility that GTP-binding proteins might be functioning on a nuclear level, perhaps in a manner analogous to Ras, to transduce growth factor receptor-coupled signals and influence nuclear function. In this report, we describe an 18 kDa nuclear protein, designated p18, which responds to growth factor treatment of cells with a greatly enhanced ability to bind GTP. The GTP-binding activity of p18 is also associated with the G_1/S phase of the cell cycle and is activated in response to UV irradiation. Initially, we suspected that p18 was a nuclear Ras-related molecule. However, through a combination of approaches, we have determined that p18 is identical to the RNA cap-binding protein, CBP20. The CBP20 protein, together with its 80 kDa binding partner, CBP80, forms a functional complex termed CBC (8-11). This nuclear complex binds cotranscriptionally to the monomethylated guanosine cap structure (m^7G) of RNA polymerase II transcribed RNAs (8, 12, 13) and has been reported to play a role in diverse aspects of RNA metabolism: it increases the splicing efficiency of cap proximal introns (8, 14-16), positively affects the efficiency of 3' end processing (17), and is required for the efficient transport of U snRNAs (10). We now show that CBC binding to GTP and m^7G capped RNAs can be regulated by extracellular factors. The implications of CBP20 functioning as a novel end-point in signal transduction highlight the importance of RNA metabolism in regulated cell growth.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions

Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 10% horse serum, and antibiotic/antimycotic solution (Sigma Chem. Co.). All other cell types, including HeLa, BHK21, and COS-7 cells, were maintained in DMEM, with the addition of 10% fetal bovine serum and antibiotic/antimycotic solution. Prior to growth factor treatment, cells were switched to serum-free media for 40 hours. Growth factors [NGF (Gibco-BRL), heregulin β1 (residues 177-244; a generous gift from Dr. Mark Sliwkowski, Genentech), and EGF (Calbiochem), or 25% fetal bovine serum] were then added to the serum-free media in concentrations and for times indicated in the results at 37°C. Following treatment, the growth factor-containing media was removed and the cells were washed (2X) with Tris-buffered saline (TBS: 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 1.0 mM EDTA), and then lysed (see below). Cell cycle blocks were performed in HeLa cells. A G_0 block was achieved by switching to serum-free media for 22-24 hours. For G_1/S phase arrest, 2.5 mM thymidine was added to growth media for 22-24 hours. 80 ng/ml nocodazole was added to growth media for 22-24 hours to achieve arrest in M phase. After treatment, cells were collected, washed with TBS (2X), and lysed. To challenge cells with UV irradiation, the media was removed from serum-starved cells, and the cells were then exposed to UV light for 2 minutes. Following exposure, cells were replenished with serum-free media and allowed to recover at 37°C for the times indicated in the text.

Cell Fractionation and Nuclear Lysis

Tissue culture cells were washed (2X) on the plate with TBS and then lysed in a buffer containing Hank's buffer (20 mM Hepes, pH 7.4, 5 mM KCl, 137 mM NaCl, 4 mM $NaHCO_3$, 5.5 mM D-glucose, 10 μM EDTA), 0.3% (v/v) NP-40, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 μg/ml each of leupeptin and aprotinin. The

lysate was then centrifuged for 15 minutes at 800 rpm at 4°C. The resulting supernatant was micro-centrifuged for 10 minutes at 4°C, and this supernatant saved as the cytoplasmic fraction. The nuclear pellet was then washed (2X) with an equal volume of Hank's buffer with 0.2% (v/v) Triton X-100, and centrifuged for 15 minutes at 800 rpm at 4°C. The resulting pellet was treated as the purified nuclear fraction. The nuclei were then lysed in a buffer containing 50 mM Tris, pH 7.4, 1% Triton X-100 (v/v), 400 mM KCl, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitors as described above. The samples were incubated on ice for 30 minutes, microfuged for 10 minutes at 4°C, and the resulting supernatant was used as the whole nuclear fraction. For nuclear fractionation, nuclei were isolated from tissue culture cells, and nuclear membranes and nuclear soluble fractions were then prepared as described by Davis and Blobel (18) with some modification. The whole nuclear fraction was resuspended in 50 mM Tris-HCl, pH 7.4, 10% (w/v) sucrose, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM $MgCl_2$, and protease inhibitors. DNase-1 (5 mg/ml) and RNase A (1 mg/ml) were added, and the nuclei were then incubated for 15 minutes at 37°C. Following the incubation with DNase-1, the nuclei were underlayed with 30% sucrose and then subjected to centrifugation in a swinging bucket rotor for 10 minutes at 20,000*g* to generate a soluble nuclear fraction and a nuclear membrane fraction.

Photoaffinity Labeling of GTP-Binding Proteins

Photoaffinity labeling of GTP-binding proteins with [α^{32}P]GTP was performed as previously described (19). In brief, the UV crosslinking reaction was carried out in a buffer that contained 50 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 µM AMP-PNP. Samples (20 µl) prepared from the cell fractionation procedures, described above, were incubated for 10 minutes at room temperature with an equal volume of crosslinking buffer containing [α^{32}P]GTP (2-3 µCi per sample) (3000 Ci/mmol, New England Nuclear) in a 96 well, non-tissue culture-treated plate. The samples were then placed in an ice bath and irradiated with UV light (254 nm) for 15 minutes. After irradiation, samples were mixed with

5X Laemmli buffer and boiled. SDS-PAGE was performed using 15% acrylamide gels. The gels were then typically Silver-stained, dried, and autoradiography was performed (typically overnight) using Kodak X-OMAT XAR-5 film at -80°C. To perform competition experiments, competing nucleotides (m^7GpppG, GpppG [New England Biolabs], m^7GTP, GTP, GMP-PNP, AMP-PNP [Sigma]) were added to the sample prior to the addition of the [α^{32}P]GTP-containing crosslinking buffer. This buffer did not contain AMP-PNP. The samples were then subjected to UV-crosslinking as described above.

Molecular Size Determination of Native p18

Soluble nuclear lysate was prepared as described above from asynchronously growing PC12 cells. Approximately 18 mg of total lysate protein were loaded onto a FPLC, Superdex 200 Hiload 16/60 column (Pharmacia) in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA (Buffer A) at a flow rate of 0.5 ml/min. Two ml fractions were collected and 30 µl of each fraction were assayed for the incorporation of [α^{32}P]GTP as described above, with the addition of 20 mM EDTA to compensate for the presence of 10 mM $MgCl_2$ in the gel filtration buffer. For gel filtration of nuclear lysates preloaded and UV-crosslinked with [α^{32}P]GTP, PC12 cell nuclear lysates were prepared (approximately 15 mg total protein) in a volume of 1.5 ml, containing 10 mM EDTA. The lysates were assayed in 6 well tissue culture plates, with 500 µl of lysates, 333 µl of UV-crosslinking buffer, and 40 µCi [α^{32}P]GTP per well. After UV-crosslinking, the lysate (2.5 ml) was applied to a PD-10 column (Pharmacia) to remove excess [α^{32}P]GTP and eluted with 3.5 ml of buffer A. The UV-crosslinked lysate was then subjected to gel filtration as described above. Following fractionation, 30 µl of each fraction was subjected to SDS-PAGE and autoradiography.

Purification of p18 from Bovine Retinal Tissue

Bovine retina were obtained frozen from J.A. & W.L. Lawson Co. (Lincoln, NE). The retina (typically 200/batch) were thawed in a buffer (TKM) containing 50 mM Tris, pH 8.0, 25

mM KCl, and 5 mM $MgCl_2$, together with protease inhibitors as described for cell lysate preparations, and then homogenized with a motor-driven dounce homogenizer. The homogenate was then centrifuged at 2500 rpm in a swinging-bucket rotor to yield a crude nuclear pellet. The nuclei were purified from this crude preparation using the method described by Blobel and Potter (20) and the soluble nuclear contents were then extracted as described above. p18 GTP-binding activity was precipitated using 40-75% ammonium sulfate, resuspended in 3-5 ml of Buffer A, and loaded onto a FPLC Superdex-200 Highload 16/60 column as described above. The purification of p18 was monitored both by Silver-staining and UV-crosslinking to [α^{32}P]GTP. The fractions eluted from the Superdex-200 column were assayed for [α^{32}P]GTP-binding to p18, and 6 peak fractions (eluting with molecular mass ~100-150 kDa) were pooled in a final volume of 12 ml and loaded directly onto a FPLC ion exchange, Mono Q 5/5 (Pharmacia) column equilibrated in Buffer B (Buffer A minus KCl). Bound proteins were eluted from the Mono Q 5/5 with a 28 ml linear gradient of 100 mM - 500 mM NaCl. p18 [α^{32}P]GTP-binding activity elutes from the Mono Q 5/5 column with ~ 300 mM NaCl in a volume of 5 ml. Peak p18 [α^{32}P]GTP-binding activity eluted from the Mono Q column was applied directly to a Bio-Gel HPHT hydroxyapatite column (Biorad) equilibrated in Buffer C (10 mM potassium phosphate, pH 6.8, 2.5 mM $MgCl_2$, 0.01 mM $CaCl_2$, 1 mM DTT). Bound proteins were then eluted, first by stepping the potassium phosphate to 100 mM, and then by a 20 ml linear gradient of 100 mM - 300 mM potassium phosphate. p18 [α^{32}P]GTP-binding activity was found to elute with ~250 mM phosphate.

Cloning and Expression of Recombinant CBP20

CBP20 was cloned by PCR from HeLa cell cDNA (a generous gift from Dr. Wannian Yang, Cornell University). 5' and 3' primers were designed using the published sequence for hsCBP20 (accession# P52298), and the CBP20 gene was then amplified from the HeLa cell cDNA using 40 PCR cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C). The 470 base product was inserted into a cloning vector, pCR2.1, using a TA cloning kit (Invitrogen), and then subcloned

into the mammalian expression vector, pcDNA3 (Invitrogen), and into the pGEX-2TK *E. coli* expression vector.

E. coli transformed with pGEX-2TK-CBP20 vector were grown in a one liter culture, and expression of glutathione-S-transferase (GST)-CBP20 protein was induced for three hours using IPTG. Following induction, the cells were pelleted by centrifugation (5000 rpm for 10 minutes in a JA-10 rotor). The harvested cells were resuspended in 15 ml of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1 mM DTT, and protease inhibitors (as described above), and then lysed using 15 mg of lysozyme, followed by the addition of 200 mM $MgCl_2$ and 1 mg DNase-1. Following centrifugation (100,00 x *g* for 30 min at 4°C), the supernatant was incubated with glutathione agarose beads for 1 hour at 4°C to bind the GST-CBP20. The glutathione agarose-bound CBP20 was washed with 50 mM Tris-HCl, pH 8.0, 0.5% (v/v) Triton X-100, 200 mM KCl , and 1 mM DTT, and then stored in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 μM GTP and protease inhibitors. GST-CBP20 was eluted from the glutathione agarose beads using 10 mM glutathione, pH 8.0, and the GST moiety was cleaved from CBP20 by the addition of 500 U thrombin for 30 minutes at room temperature.

Using a lipofectamine protocol (Gibco-BRL), a hemaglutinin-tagged form of CBP20 (HA-CBP20) was transiently transfected into BHK21 cells according to the manufacture's directions. Following a 5 hour incubation with serum-free media containing the lipid/DNA complex, the media was removed and replaced with media containing 10% fetal bovine serum. Cells were allowed to grow in the presence of serum for approximately 20 hours and were then switched to serum-free media for 40 hours prior to stimulation with serum.

Immunoprecipitation and Western Immunoblotting

A polyclonal antibody generated against recombinant CBP80 (αCBP80 (8)) and a monoclonal antibody recognizing the hemaglutinin epitope were prepared as previously described. Cytosolic and nuclear lysates were prepared as described above. Prior to immunoprecipitation, the cytosolic lysate was adjusted to 100 mM NaCl and the nuclear lysate was diluted three fold with 50

mM Tris-HCl, pH 8.0, 1 mM DTT, and 1 mM sodium orthovanadate. The lysates were then allowed to incubate at 4°C for one hour, with or without the addition of 5 µl of 12CA5 mAb or αCBP80 pAb. Following the first incubation, 40 µl of protein-A Sepharose beads were added to each sample and the samples were incubated for another hour at 4°C. The samples were then centrifuged and the immunoprecipitated pellets were washed 4 times with 50 mM Tris-HCl, pH 8.0, 133 mM KCl, 0.33% Triton X-100, 1 mM DTT, and 1 mM sodium orthovanadate. The resulting immunoprecipitated pellets were resuspended in 20 µl of UV-crosslinking buffer and subjected to incubation with [α^{32}P]GTP and UV-crosslinking as described above.

For Western blotting analysis, proteins were transferred to PVDF membranes following SDS-PAGE. The PVDF membranes were blocked with 2.5% (w/v) BSA in Tris-buffered saline (TBS) plus 0.1% Tween-20 for one hour at room temperature. After blocking, the membranes were incubated with either 12CA5 or αCBP80 antibody for one hour at room temperature, washed with several changes of TBS-0.1% Tween-20, and incubated for thirty minutes at room temperature with sheep-anti-rabbit or sheep-anti-mouse horseradish peroxidase-conjugated antibody (Amersham), as appropriate, and washed. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

RNA-Binding Assays

UV-crosslinking was done essentially as described by Rozen and Sonenberg (21) except that the RNA probe was transcribed from BamHI-cleaved pBSII KS with T3 RNA polymerase (Promega).

RESULTS

Identification of a nuclear, growth factor-responsive GTP-binding activity

In attempts to identify novel growth factor-responsive GTP-binding activities, HeLa cells were first serum-starved and then treated with epidermal growth factor (EGF) and heregulin [the ligand for the Neu/Erb2:ErbB3 receptor heterodimer (22, 23)]. Following growth factor treatment for 15 minutes, the cells were then fractionated and the resulting cytoplasmic and nuclear lysates were assayed to determine if any protein showed a change in its ability to bind [α^{32}P]GTP, as assayed by UV-crosslinking (see "Experimental Procedures"). As shown in Figure 1A (left panel), nuclear lysates from HeLa cells contained a GTP-binding activity that corresponded to a protein with an apparent M_r of ~18 kDa. This activity was strongly stimulated by heregulin and to a lesser extent by EGF. By nuclear fractionation we determined that the growth factor-stimulated GTP-binding activity was associated with the soluble nuclear contents, but not with the nuclear membrane or nuclear pore (data not shown). Thus far, we have observed a similar GTP-binding activity in every cell line that we have tested including PC12 cells (see below), COS-7 cells, human embryonic kidney cells (293), baby hamster kidney cells (BHK21), human epidermoid carcinoma (A431) cells, and various mammary epithelial cells (data not shown). In the case of PC12 cells (shown in Figure 1A, right panel), the GTP-binding activity corresponding to an 18 kDa nuclear protein (from here on designated as p18) was strongly stimulated by NGF, as well as by heregulin and EGF. Figure 1B shows that the growth factor-stimulated binding of [α^{32}P]GTP by p18 was dose-dependent.

The original GTP-binding assays for p18 were performed after relatively short periods of growth factor treatment (i.e. 15 minutes). While this was sufficient to detect [α^{32}P]GTP-binding by p18, more complete time course experiments indicated that near maximal GTP binding required growth factor treatment for 1 hour. An example for PC12 cells is shown in Figure 1C. In this experiment, serum-starved PC12 cells were challenged with 100 ng/ml NGF for increasing time

periods, up to 24 hours. The results show that near maximal GTP-binding was observed after approximately 1 hour of growth factor addition and that the stimulated GTP-binding activity continued through the 24 hours of treatment. A similar time course of GTP-binding to p18 was observed when PC12 cells were treated with heregulin (data not shown).

We have found that nuclear lysates from asynchronously growing cells also contained p18, suggesting that this growth factor-dependent GTP-binding activity may be associated with a particular phase of the cell cycle. To investigate this possibility, HeLa cells were arrested in G_0 by serum starvation, in G_1/S by thymidine addition, and in M phase by nocodazole treatment (Figure 1D). The cells were then fractionated into cytoplasmic and nuclear fractions (or a mitotic pellet was prepared in the case of M phase-arrested cells) and the resulting lysates were then assayed for the ability of p18 to bind [α^{32}P]GTP. As expected, we found that p18 did not bind GTP in cells arrested in G_0. p18 also did not bind GTP in either the cytoplasm or mitotic pellet of cells arrested in M phase. However, p18 did show strong GTP-binding activity in the nucleus of HeLa cells arrested in G_1/S. Thus, the GTP-binding activity of p18 appears to be subject to cell cycle-dependent, as well as growth factor-dependent regulation.

To determine whether this nuclear activity might respond to a broader range of stimuli, we determined the ability of p18 to bind GTP under conditions of cellular stress. UV light is known to elicit a stress response in cells, via the low molecular weight GTP-binding proteins Cdc42 and Rac, which culminates in transcriptional activation through the stimulation of the stress-responsive nuclear MAP kinases, the c-Jun NH_2-terminal kinase (JNK1) and p38/HOG1 (4-7). PC12 cells were therefore serum-arrested and exposed to UV light for 2 minutes. Following UV exposure, the cells were allowed to recover for 30 minutes or 1 hour and p18 was assayed for [α^{32}P]GTP-binding activity. Figure 1E shows that p18 was stimulated to bind GTP when PC12 cells were exposed to UV light. A similar UV-stimulated GTP-binding activity was observed in COS-7 and HEK-293 cells (data not shown).

Biochemical characterization of the nucleotide-binding properties of p18

Figure 2A compares the abilities of the non-hydrolyzable GTP-analog, GMP-PNP, and the non-hydrolyzable ATP-analog, AMP-PNP, to compete with [α^{32}P]GTP for binding to p18. These data show that the addition of increasing concentrations of GMP-PNP strongly inhibited the binding of [α^{32}P]GTP to p18, with half maximal inhibition occurring at ~100 μM. In contrast, AMP-PNP did not show measurable competition versus [α^{32}P]GTP until levels of 10 mM were used. A more direct comparison of nucleotide binding specificity is shown in Figure 2B. Here, nuclear lysates from either serum-starved or NGF-stimulated PC12 cells were assayed for the binding of [α^{32}P]GTP or [α^{32}P]ATP to p18. Under conditions where p18 was strongly labeled with [α^{32}P]GTP, no labeling with [α^{32}P]ATP was detected. These results suggest that p18 has a significantly higher affinity for guanine nucleotides compared to adenine nucleotides.

It has been generally assumed that small proteins (Mr $\leq$ 60 kDa) are free to diffuse into and out of the nucleus through the nuclear pores but can be retained in the nucleus (24) when they exist as part of a higher molecular weight complex. Given that we have only detected GTP-binding to p18 in the nucleus, we examined the possibility that p18 is retained in the nucleus as part of a larger protein complex. Nuclear lysates were fractionated by gel filtration chromatography and the resulting fractions were assayed for photo-catalyzed incoporation of [α^{32}P]GTP. Figure 3A shows that p18 eluted from the gel filtration column with an approximate molecular mass of 100-150 kDa. We did not find p18 activity in fractions which eluted from the gel filtration column with an approximate molecular mass of 18-20 kDa, suggesting that p18 was activated to bind [α^{32}P]GTP within an oligomeric or multi-protein complex. This raised the possibility that p18, upon nucleotide binding, might dissociate from the protein complex to yield a monomeric species. To examine this possibility, we performed a gel filtration experiment where the nuclear lysate was subjected to loading and UV-crosslinking with [α^{32}P]GTP prior to chromatography. Figure 3B shows the corresponding elution profile. Under these conditions, the [α^{32}P]GTP-labeled p18 was detected both in fractions that corresponded to an apparent of M_r of 100-150 kDa and in

fractions that corresponded to its monomeric size of 18 kDa. Together these results suggest that p18 can exist in the nucleus both as part of a higher molecular weight protein complex and as a monomeric species, but that complex formation is necessary for its GTP-binding activity.

Identification of p18 as the RNA cap-binding protein, CBP20

As a first step toward determining the molecular identity of p18, attempts were made to purify it in sufficient quantities for microsequencing analysis. A number of potential sources for purification were examined including different cell culture lines, mammalian tissues (rabbit liver, bovine brain, and bovine retina), *Xenopus* oocytes, and the yeast *S. cerevisiae.* While each of these sources contained a GTP-binding activity that co-migrated with p18, bovine retina appeared to be most highly enriched in this activity. Therefore, soluble nuclear extracts were prepared from bovine retina and following ammonium sulfate fractionation, p18 activity, as assayed by photo-catalyzed incorporation of [α^{32}P]GTP, was separated from other nuclear proteins by a series of chromatography steps (see "Experimental Procedures"). After gel filtration, ion exchange and hydroxyapatite chromatography, p18 activity was resolved from the majority of contaminating low molecular weight proteins. These steps also resolved an 80 kDa species (designated p80), detected by Silver staining, which appeared to co-purify with p18 activity (data not shown). However, these steps failed to yield a protein band (as assessed by Silver-staining) in the 18 kDa range that could be definitively aligned with bands on the autoradiographs corresponding to the incorporation of [α^{32}P]GTP.

The apparent co-purification of p18 activity with an 80 kDa protein was reminiscent of what had been reported for the 18 kDa nuclear protein, CBP20 (for cap-binding protein 20). CBP20 forms a stable complex with an 80 kDa protein, CBP80, to bind a guanine derivative, the ^{7}methylguanosine (m^{7}GpppN) cap structure on RNAs transcribed by RNA polymerase II. Heterodimerization of CBP20 and CBP80 is necessary for the cap-binding activity (8-11). Thus, the binding properties of CBP20 seemed analogous to the apparent association of p18 activity with a multi-protein complex and its co-purification with p80. In addition, CBP20 can be covalently

crosslinked to capped RNAs using a UV-crosslinking procedure similar to the one employed in this study (8). To investigate the possibility that p18 might correspond to CBP20, fractions (from the three chromatography steps described above) containing peak p18 activity were analyzed for the presence of both the CBP20 and CBP80 proteins by Western blotting with specific anti-CBP20 and anti-CBP80 polyclonal antibodies. The results from this analysis revealed that peak p18 fractions, obtained following three successive chromatography steps, were enriched in both CBP20 and CBP80. Figure 4B shows the Western blot analysis for CBP20 and CBP80 that was performed on hydroxyapatite fractions containing peak p18 activity (Figure 4A).

Additional experiments were then performed to confirm the identity of p18 as CBP20. First, we examined whether p18 was capable of binding RNA cap analogs such as m^7GpppG. In Figure 4C, different cap analogs (m^7GpppG, GpppG, m^7GTP), as well as GTP, were used to compete with the binding of [α^{32}P]GTP to p18 partially purified from bovine retina. At concentrations of 10 μM, all three cap analogs completely abolished the binding of [α^{32}P]GTP to p18, while GTP competed with approximately 80% efficiency. Thus, the bovine retinal p18, like CBP20, was capable of binding cap-analogs and in fact, appeared to bind these analogs with a higher affinity than GTP.

We next examined the ability of the RNA cap-analogs to compete with [α^{32}P]GTP for binding to p18 in PC12 cells, where a growth factor-stimulated GTP-binding response had previously been observed. To add an additional level of stringency, PC12 cell nuclear lysates were first immunoprecipitated with antibodies generated against CBP80 (i.e. the binding partner of CBP20) and these immunoprecipitates were then assayed for photo-catalyzed incorporation of [α^{32}P]GTP to p18 in the absence and presence of RNA cap-analogs or GTP. As shown in Figure 4D, the [α^{32}P]GTP-binding activity corresponding to the endogenous p18 was co-immunoprecipitated with CBP80 from asynchronously growing PC12 cells. This activity was effectively inhibited by the addition of low concentrations of cap-analogs to the [α^{32}P]GTP crosslinking assay, thus yielding the following binding specificity:

$m^7GpppG > m^7GTP > GpppG > GTP$ (Figure 4E). These data are in accord with the binding specificity for different cap analogs to the CBC reported by Izaurralde et. al. (1992). Therefore, p18 isolated from PC12 cell nuclear lysates was able to form a complex with CBP80 and bind cap analogs in a manner identical to CBP20.

We then set out to confirm that *E. coli*-expressed GST-CBP20 or the recombinant CBP20/CBP80 complex (CBC) (see "Experimental Procedures") can be UV-crosslinked to [α^{32}P]GTP (i.e., as originally observed for p18). Figure 5A shows the GST-CBP20, thrombin-cleaved CBP20, and the CBC proteins (as visualized by Coomassie blue staining) which were assayed for [α^{32}P]GTP-binding. The corresponding autoradiogram in Figure 5B shows that the recombinant CBP20 proteins were able to bind [α^{32}P]GTP in the UV-crosslinking assay. This binding was greatly enhanced by the presence of CBP80 (see lane 5). The GST control did not show any crosslinking to [α^{32}P]GTP. Thus, based on a number of criteria, we conclude that p18 and CBP20 have identical biochemical properties.

Evidence for a regulated GTP and capped-RNA binding by the CBC

To ascertain whether CBP20, like p18, could be regulated by extracellular stimulation, a hemaglutinin (HA)-tagged CBP20 construct was transiently transfected into BHK21 cells. Following 40 hours of serum starvation, these transiently transfected cells were stimulated with 25% fetal bovine serum for 1.5 hours, and HA-CBP20 was immunoprecipitated from the cytosolic and nuclear lysates prepared from either serum-starved or stimulated cells. The immunoprecipitated HA-CBP20 was then assayed for [α^{32}P]GTP binding. HA-CBP20 was present in both the cytosolic and nuclear fractions (Figure 6B), and CBP80 co-immunoprecipitated with the nuclear localized HA-CBP20 equally well under conditions of either serum starvation or stimulation (Figure 6A). The large percentage of HA-CBP20 localized to the cytosol is presumably the result of its over-expression. However, the cytosolic HA-CBP20 did not show

any detectable binding to [α^{32}P]GTP (Figure 6C), whereas, the nuclear HA-CBP20 showed a marked serum-stimulated incorporation of [α^{32}P]GTP.

We next examined whether the growth factor regulation of CBP20 extended to the binding of capped RNAs. Figure 7A (left panel) shows the typical [α^{32}P]GTP-binding response by p18 in nuclear lysates prepared from PC12 cells which were serum-starved, and then stimulated with NGF for 15 minutes. When m^7G^{32}pppN-capped RNA was used as a substrate in the UV-crosslinking assay instead of [α^{32}P]GTP (see "Experimental Procedures"), an essentially identical binding pattern was observed (Figure 7A, right panel). Figure 7B shows that identical results were also obtained with PC12 cells which stably express HA-tagged CBP20. Specifically, NGF strongly stimulated both the [α^{32}P]GTP binding and the m^7G^{32}pppN-capped RNA binding by HA-CBP20 that was precipitated from the nuclear lysates with the 12CA5 antibody.

DISCUSSION

The original goal of these studies was to identify novel nuclear activities that were susceptible to growth factor regulation, in order to further our understanding of how growth factors exert their effects in the nucleus. Our initial emphasis was on GTP-binding activities given their roles as tightly regulated molecular switches which mediate a diversity of receptor-coupled signaling systems, both at the cell surface and in the cytosol. Using a photoaffinity labeling approach to detect nuclear proteins that specifically incorporate [α^{32}P]GTP, we detected an 18 kDa nuclear activity which was highly sensitive to the addition of growth factors to G_0 arrested cells. The fundamental role of this activity in cell growth regulation is underscored by its response to growth factors, its specific association with the G_1/S phase of the cell cycle, its activation under conditions of cell stress, and the fact that we have found this activity in every cell and tissue type examined thus far. It was therefore interesting to find that the 18 kDa activity corresponds to the RNA cap-binding protein, CBP20, suggesting a necessity for a regulated nuclear cap-binding event in cell growth control.

The m^7G(5')ppp(5')N cap structure on RNAs transcribed by RNA polymerase II has been known for some time to be important for the stability of these RNAs (25, 26), as well as to facilitate different aspects of RNA metabolism including translation initiation, pre-mRNA splicing, and nuclear transport. In recent years CBP20 and its 80 kDa binding partner, CBP80, (collectively termed CBC) have been identified as the protein complex which binds to the cap structure in the nucleus and mediates the cap effect on pre-mRNA splicing and export of U snRNAs (8, 10). To our knowledge, this is the first report describing a regulated binding activity by the CBC and thus implies that RNA metabolic processes ascribed to the CBC will be regulated as well. While our initial identification of the CBC as a downstream target of cellular signal transduction pathways was based on the ability of CBP20 to bind [α^{32}P]GTP in a photoaffinity labeling assay, it is likely, based on our competition experiments and those of others (12), that the main substrate for

the CBC is indeed capped RNA. However, while the affinity of the CBC for GTP is weaker than what would be anticipated for a conventional GTP-binding protein, we cannot discount the possibility that the CBC binds GTP *in vivo*.

An understanding of the signaling processes which lead to CBC activation could shed light on how mitogens influence gene expression by modulating RNA metabolism.. All indications are that the CBC may receive inputs from multiple pathways. The Ras-Raf-MEK-ERK signaling cascade is one pathway which is central to mediating growth factor effects in the nucleus and represents an obvious possible mechanism for growth factor-stimulated CBC activation. Additionally, we are investigating the involvement of stress-activated signaling pathways in CBC activation, since we have found that CBP20 responds to UV-light exposure as well as growth factor treatment (Figure 1E). Of particular interest is the pathway that begins with the Cdc42 or Rac GTP-binding proteins and results in the stimulation of the stress-activated nuclear MAP kinases JNK1 and p38 (4-7, 27). There are a number of lines of evidence which indicate that signaling pathways stimulated by Rho-like GTP-binding proteins (e.g. Cdc42, Rac) are also under growth factor control (28-30). Our preliminary data suggest that CBC activity is elevated in response to activated forms of Ras and Cdc42 (K.F. Wilson, unpublished data), supporting a role for multiple signaling pathways in the regulation of the CBC.

Alternatively, mitogenic signaling pathways involved in translational control, such as a rapamycin-sensitive pathway involving FRAP (FKB12-rapamycin-associated protein) and/or S6 kinase, may input into CBC activation. This is particularly relevant given the findings that the cytosolic mRNA cap-binding protein, eIF-4E, is also susceptible to growth factor regulation. eIF-4E, along with eIF-4A, eIF-4B, and eIF-4G, comprise the translation initiation complex (eIF-4F) which binds capped mRNA (reviewed in 29). Phosphorylation of eIF-4E occurs in response to multiple growth factors (including NGF in PC12 cells) and cell cycle arrest (31)(30), and appears to occur downstream of multiple signaling pathways including the ERK, JNK/SAPK and p38

kinase pathways (32). In addition to its direct phosphorylation, the activity of eIF-4E is also regulated by two other growth factor-responsive factors, the eIF-4E binding proteins 4E-BP1 and 4E-BP2 (33). The phosphorylation of 4E-BP1 is downstream of a rapamycin-sensitive pathway distinct (34) from the pathways culminating in eIF-4E phosphorylation. Recently, 4E-BP1 has shown to be phosphorylated by the PI 3-K-related kinase, FRAP (35). Thus, it will be interesting to see if the cytosolic cap-binding protein, eIF-4E, and the CBC are similarly, or even coordinately regulated, thereby establishing a functional interplay between cap-dependent nuclear mRNA processing, transport, and translation.

Neither the cellular levels of CBP20, nor its ability to bind CBP80 or its nuclear localization are affected by growth factor simulation (see Figure 6), suggesting that the change in activity displayed by the CBC in response to growth factors must be achieved via an alternate mechanism. There is evidence for post-translational modification (including phosphorylation and methylation) of the CBC, and CBP20 has been found to be phosphorylated *in vivo* (data not shown). A growth factor-dependent phosphorylation of CBP20 or CBP80 could have a direct effect on the RNA cap-binding activity of CBP20, perhaps in a manner similar to the effect of phosphorylation on the cytosolic cap-binding protein, eIF-4E. Alternatively, growth factors might influence the interactions between the CBC and specific regulatory proteins in order to stimulate the binding of the CBC to capped RNA-binding (i.e., in a manner analogous to the growth factor-regulated interaction between eIF-4E and the 4E-BPs). Identification of such kinases and/or binding partners for the CBC will be important in defining nuclear signaling pathways involved in RNA processing control.

Our demonstration that the CBC is susceptible to extracellular regulation, in conjunction with the previously defined role for the CBC in RNA processing, make the CBC an attractive candidate for translating growth factor-signals into altered gene expression by affecting the metabolism of specific subsets of RNAs. However, given that the CBC affects both the

processing and transport of RNAs transcribed by RNA polymerase II, the growth factor-dependent binding of the CBC to capped RNA may result in a general regulation of gene expression. The reduced ability of the CBC to bind capped RNAs in the absence of a growth factor signal could serve as a checkpoint for cell growth by guarding against the further processing of inappropriate or "leaky" transcripts. This suggests that altered levels and/or mutations of the CBC might be capable of deregulating cell growth. Future studies will be directed toward determining how growth factors influence different aspects of RNA processing (including precursor mRNA splicing and RNA export) through the CBC, and how over-expression and/or mutation of the CBC impacts upon normal cell growth.

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FIGURE LEGENDS

Figure 1. p18 is an 18 kDa nuclear protein whose GTP-binding is stimulated by multiple stimuli including growth factors, G_1/S arrest, and cell stress.

(A) HeLa cells (lanes 1-6) were serum-starved (control lanes 1 and 4) and then treated with 100 ng/ml EGF (lanes 2,5) or 30 nM heregulin (HRG) (lanes 3, 6) for 15 minutes at 37°C. PC12 cells (lanes 7-14) were serum-starved (control lanes 7 and 11) and then treated with 30 nM HRG (lanes 8, 12), 100 ng/ml EGF (lanes 9, 13), and 100 ng/ml NGF (lanes 10, 14). The cells were then lysed and separated into cytoplasmic (lanes 1-3, 7-10) and whole nuclear (lanes 4-6, 11-14) fractions and assayed for [α^{32}P]GTP-binding by UV-crosslinking using 45 μg of protein from cell lysates.

(B) A dose response experiment was performed using the addition of either heregulin (lanes 2-4) or NGF (lanes 5-7) to serum-starved PC12 cells (control, lane 1) for 30 minutes at 37°C. For each dose of heregulin or NGF, 50 μg of total nuclear lysate protein were assayed for GTP-binding by UV-crosslinking with [α^{32}P]GTP and then 15% SDS-PAGE was performed. The resulting gel was dried and exposed to X-ray film for 5-15 h.

(C) A time course of 100 ng/ml NGF treatment was performed in serum-starved PC12 cells (control, lane 1) with NGF addition for 7.5 minutes (lane 2), 15 minutes (lane 3), 30 minutes (lane 4), 60 minutes (lane 5), or 24 h (lane 6). 50 μg of protein from the nuclear lysates were assayed for binding to [α^{32}P]GTP by UV-crosslinking, followed by 15% SDS-PAGE and autoradiography overnight.

(D) HeLa cells were arrested in G_0 by serum starvation (lanes 1,4) in G_1/S by 2.5 mM thymidine addition (lanes 2,5) and in M phase with 80 ng/ml nocodazole (lanes 3,6). Cells were then separated into cytoplasmic (lanes 1,2,3) and whole nuclear fractions (or a mitotic pellet was prepared for M phase arrest) (lanes 4,5,6) and then for each fraction, 50 μg of protein were assayed for [α^{32}P]GTP-binding activity by UV-crosslinking followed by 15% SDS-PAGE and autoradiography.

(E) PC12 cells were serum-starved and then exposed to UV light for 2 minutes. Following exposure, cells were replenished with serum-free medium and allowed to recover for 30 minutes or one hour. Cells were then harvested, nuclear lysates were prepared, and 50 μg of nuclear lysate protein were assayed for [α^{32}P]GTP-binding by UV crosslinking followed by 15% SDS-PAGE and autoradiography.

Figure 2. The nucleotide binding activity of p18 is specific for guanine nucleotides.

(A) 50 μg of protein from DNase-1-digested, soluble nuclear lysates from asynchronously growing PC12 cells were assayed for [α^{32}P]GTP incorporation in the presence of increasing concentrations of cold AMP-PNP [10 μM (lane 1), 50 μM (lane 2), 100 μM (lane 3), 500 μM (lane 4), 1 mM (lane 5), 5 mM (lane 6), and 10 mM (lane 7)] or in the absence (lane 8) or presence of cold GMP-PNP [5 μM (lane 9), 10 μM (lane 10), 50 μM (lane 11), 100 μM (lane 12), 500 μM (lane 13), 1 mM (lane 14), 5 mM (lane 15), and 10 mM (lane 16)]. Following UV-crosslinking, samples were separated by 15% SDS-PAGE and the resulting gel was dried and exposed to X-ray film overnight.

(B) Serum-starved PC12 cells were treated with 100 ng/ml NGF for 1 hour at 37°C. 65 μg of protein from nuclear extracts from serum-starved cells (lanes 1,3) or NGF-treated cells (lanes 2,4) were incubated and crosslinked in the presence of [α^{32}P]GTP (lanes 1,2) or [α^{32}P]ATP (lanes 3,4). Samples were separated by 15 % SDS-PAGE and the resulting gel was dried and exposed to X-ray film overnight.

Figure 3. p18 exists in the nucleus both as part of a high molecular weight complex competent to bind GTP and as a monomer.

(A) 18 mg of protein from DNase-1-digested, soluble nuclear extract from PC12 cells were fractionated by Superdex 200 chromatography (2 ml fractions were collected). 30 μl of each fraction were then subjected to UV-crosslinking with [α^{32}P]GTP in the presence of 10 μl crosslinking buffer and 20 mM EDTA, followed by 15% SDS-PAGE and autoradiography.

(B) 15 mg of protein from DNase-1-digested, soluble nuclear extract from PC12 cells were subjected to UV-crosslinking with [α^{32}P]GTP in the presence of 1 ml crosslinking buffer and 10 mM EDTA. Following crosslinking, unbound [α^{32}P]GTP was removed from the lysate using a PD10 column. The crosslinked lysate was then applied to a Superdex 200 column and 2 ml fractions were collected. 30 μl of each fraction were then separated by 15% SDS-PAGE. The resulting gel was dried and exposed to X-ray film for 8 days to visualize the elution of [α^{32}P]GTP-crosslinked proteins. Gel filtration standards are from Biorad.

Figure 4. p18 GTP-binding activity corresponds to the nuclear cap-binding protein CBP20.

(A) UV-detection of protein elution from a hydroxyapatite column. Fractions marked #13-16 correspond to peak p18 GTP-binding activity.

(B) i.) Western blot detection of CBP80 in fractions #13-16 from hydroxyapatite chromatography. ii.) Western blot detection of CBP20 in hydroxyapatite fractions #13-16. iii.) Assay for [α^{32}P]GTP-binding activity in fractions #13-16 from hydroxyapatite chromatography.

(C) Determination of guanine nucleotide specificity of p18 purified from bovine retina. Partially purified bovine retinal p18 was assayed for [α^{32}P]GTP incorporation by UV-crosslinking in the absence (lanes 1, 6, 11) or presence of increasing concentrations (10 μM, 50 μM and 100 μM) of the RNA cap-analogs, m^7GpppG (lanes 2, 7, 12), GpppG (lanes 3, 8, 13), and m^7GTP (lanes 4, 9, 14), or in the presence of GTP (lanes 5, 10, 15). Following UV-crosslinking, proteins were separated by 15% SDS-PAGE and [α^{32}P]GTP-binding activity was visualized by autoradiography.

(D) p18 [α^{32}P]GTP-binding activity co-immunoprecipitates with CBP80 from PC-12 cells. Nuclear extracts were prepared from PC-12 cells growing asynchronously in culture. Two hundred micrograms of lysate were then immunoprecipitated with either 5 μl of preimmune serum (lane 2) or with 5 μl of αCBP80 antiserum (lane 3). The immunoprecipitates (resuspended in 30 μl of UV-crosslinking buffer), or 100 μg of protein from the nuclear extract (lane 1) were then assayed for [α^{32}P]GTP-binding by UV crosslinking (lower panel). Endogenous CBP20 protein was detected by Western blotting using a specific CBP20 antiserum (upper panel).

(E) [α^{32}P]GTP-binding to p18 from PC12 cells is blocked by the addition of RNA cap analogs. CBP80 was immunoprecipitated from asynchronously growing PC12 cells as described in (D). The immunoprecipitates were then assayed for [α^{32}P]GTP-binding to p18 in the presence of: m^7GpppG (0 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, lanes 1-6), GpppG (0 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, lanes 7-12), m^7GTP (0 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, lanes 13-18), and GTP (0 μM, 0.5 μM, 1 μM, 5 μM, 10 μM, 50 μM, lanes 19-24). Following crosslinking, proteins were separated by 15% SDS-PAGE and the gel was dried and autoradiography was performed.

Figure 5. Recombinant, *E. coli*-expressed CBP20 binds and is UV-crosslinked to [α^{32}P]GTP.

CBP20 was expressed and purified from *E. coli* as a GST fusion protein. GST-CBP20 (lanes 1 and 2), free CBP20 (the GST was cleaved with thrombin) (lanes 3 and 4), or *E.coli*-expressed CBC (complex proteins designated as CBP80* and CBP20*, lane 5) were then assayed for their ability to be UV-crosslinked to [α^{32}P]GTP. Following UV-crosslinking, proteins were separated by 15% SDS-PAGE and visualized by Coomassie Blue staining (Panel A) and autoradiography (Panel B).

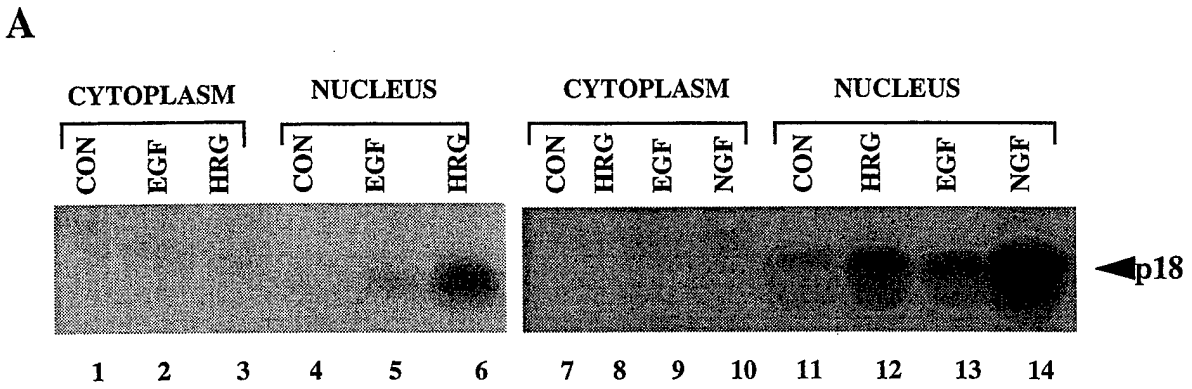
Figure 6. Serum-dependent binding of [α^{32}P]GTP to recombinant CBP20 expressed in BHK21 cells.

Human CBP20 was cloned by PCR from HeLa cell cDNA and then subcloned into the mammalian expression vector, pcDNA3, to express a hemaglutinin (HA)-tagged form of the protein. BHK21 cells were transiently transfected with HA-CBP20 (using 8 μg DNA per 100 mm plate of BHK21 cells). The transfected cells were serum-starved for 40 hours (-) and then stimulated with 25% fetal bovine serum (+) for 1.5 hours. HA-CBP20 was immunoprecipitated from cytosolic or nuclear lysates using the 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for [α^{32}P]GTP binding by UV-crosslinking. Proteins were separated by 15% SDS-PAGE and transferred to immobilon for Western blot analysis and autoradiography. Panel A shows the CBP80 protein co-immunoprecipitating with HA-CBP20 primarily from the nuclear lysates as detected by Western blotting using a CBP80 anti-serum. Panel B is a Western blot using the 12CA5 antibody to detect the immunoprecipitated HA-CBP20 from cytosolic and nuclear lysates. The [α^{32}P]GTP-binding activity corresponding to the immunoprecipitated HA-CBP20 is shown in Panel C.

Figure 7. CBC binding to capped-RNA is regulated by growth factors.

(A) UV-crosslinking of p18 to capped-RNA is stimulated by growth factors. PC12 cells were serum starved (-) and treated with NGF (100 ng/ml [+]) for 15 minutes. The cells were harvested and nuclear extracts were prepared and assayed for the ability of p18 to be UV-crosslinked to either [α^{32}P]GTP or m^7G^{32}pppG-capped RNA.

(B) CBP20 binds capped RNA in a growth factor-dependent manner. PC12 cells stably expressing a hemmaglutinin-tagged (HA) CBP20 were serum-starved (-) and then treated with NGF (100 ng/ml, 1 hr [+]). After preparation of cytosolic and nuclear lysates, HA-CBP20 was immunoprecipitated from the lysates and UV-crosslinked in the presence of either [α^{32}P]GTP (lanes 1-4) or m^7G^{32}pppG-capped RNA (lanes 5-8).


A
CYTOPLASM
NUCLEUS
CYTOPLASM
NUCLEUS
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HRG
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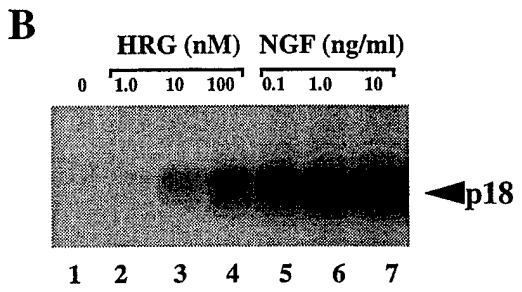
B
HRG (nM)
NGF (ng/ml)
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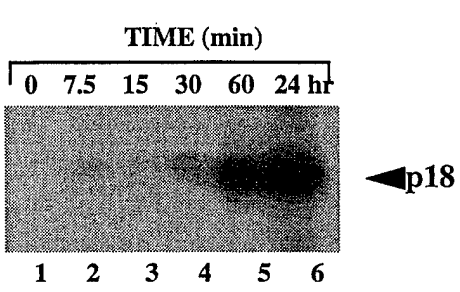
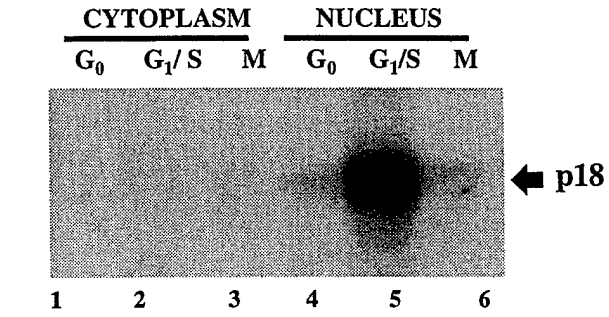
10
100
0.1
1.0
10
p18
1 2 3 4 5 6 7


C
TIME (min)
0
7.5
15
30
60
24 hr
p18
1 2 3 4 5 6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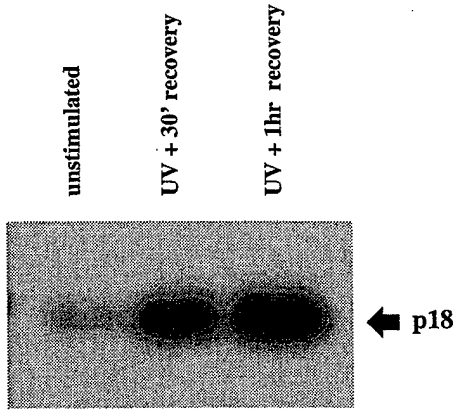
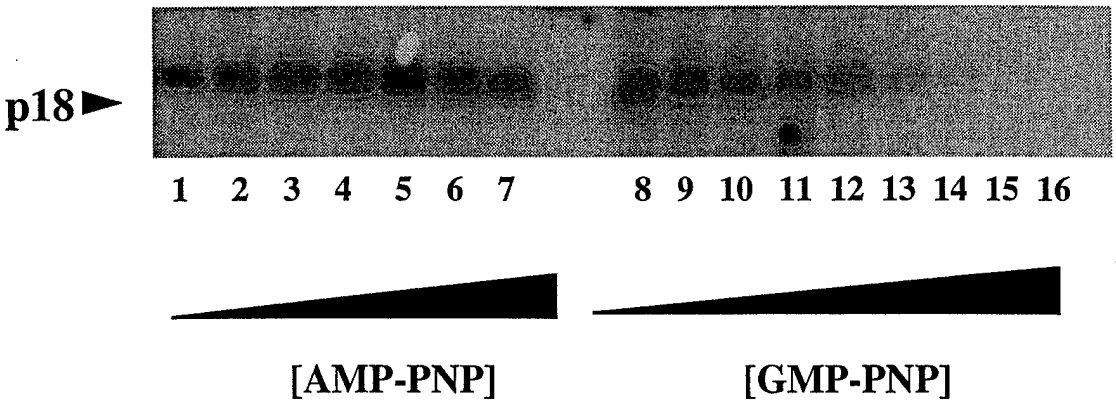
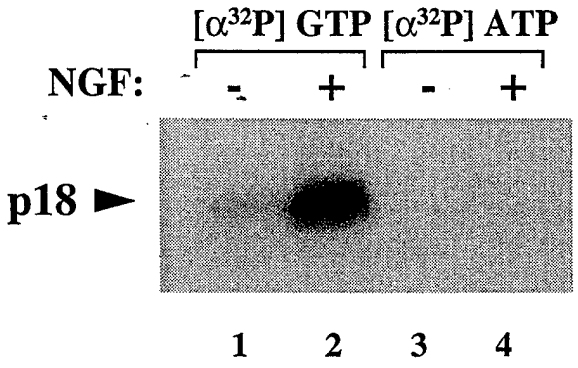
G1/S
M
G0
G1/S
M
p18
1 2 3 4 5 6


E
unstimulated
UV + 30' recovery
UV + 1hr recovery
p18

A


p18
1 2 3 4 5 6 7
8 9 10 11 12 13 14 15 16
[AMP-PNP]
[GMP-PNP]

B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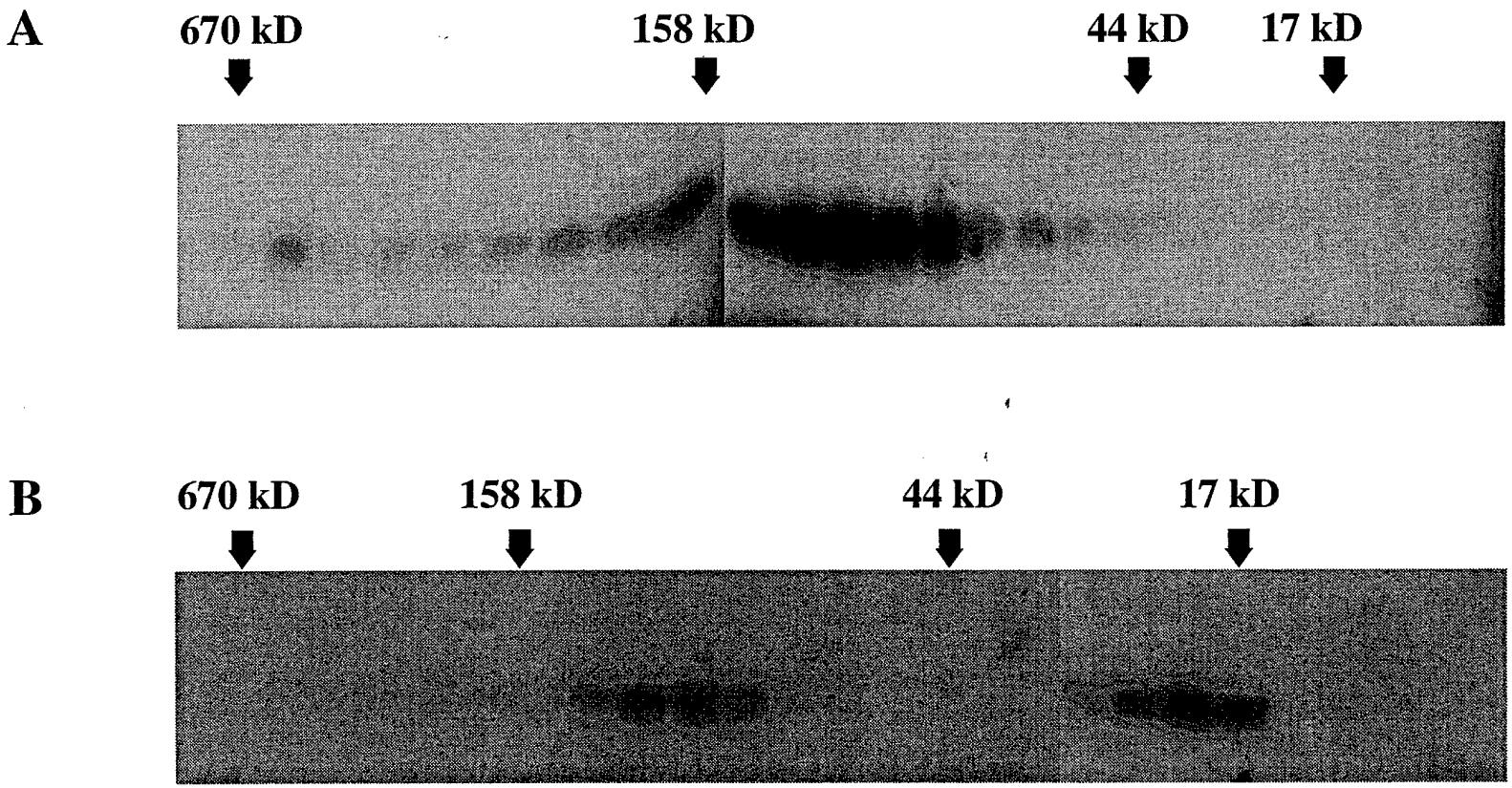
[α32P] ATP
NGF:
- + - +
p18
1 2 3 4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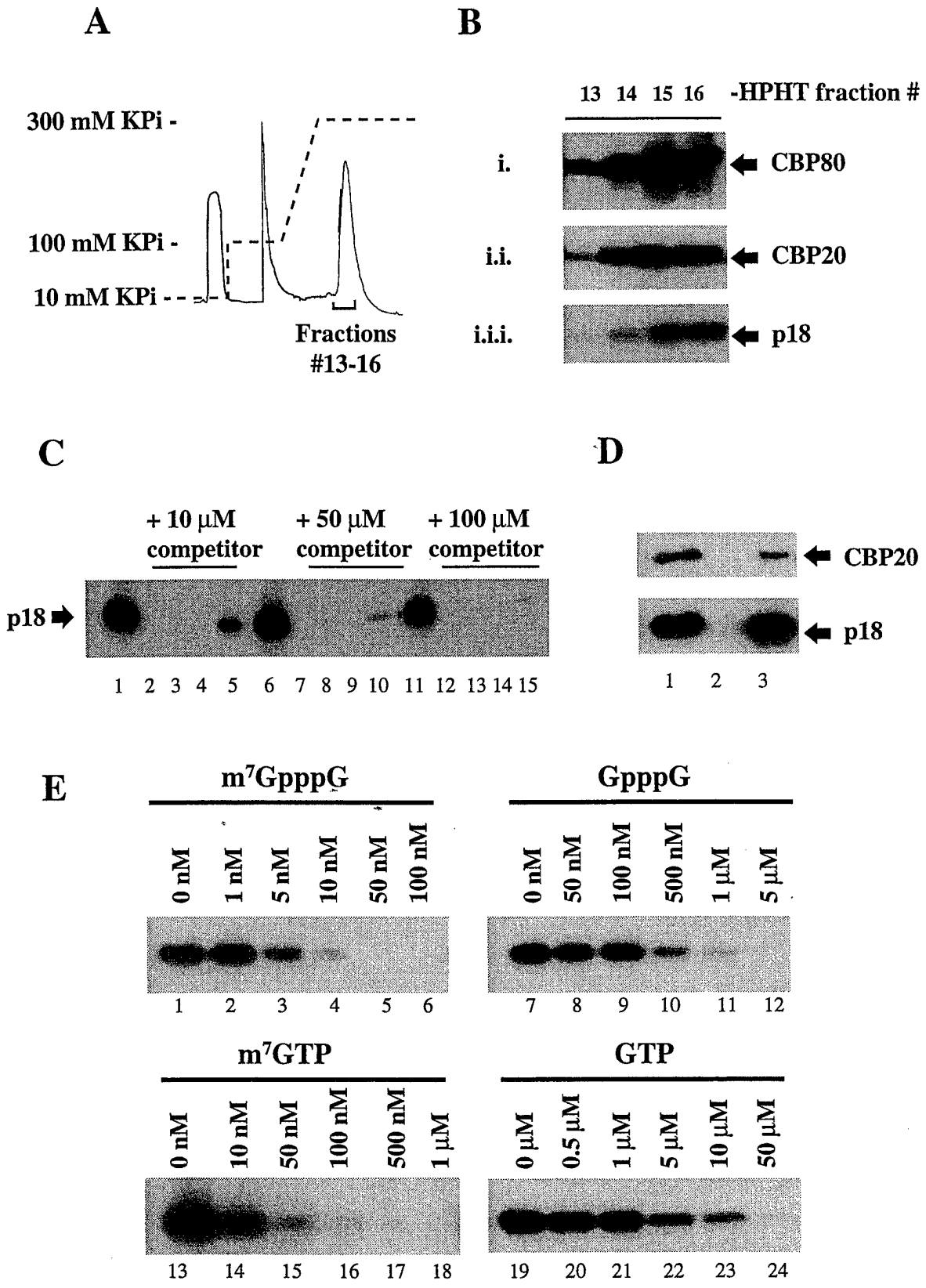
44 kD
17 kD
B
670 kD
158 kD
44 kD
17 kD


A
300 mM KPi -
100 mM KPi -
10 mM KPi - -
Fractions
#13-16
B
13 14 15 16 -HPHT fraction #
i.
CBP80
i.i.
CBP20
i.i.i.
p18
C
+ 10 μM competitor
+ 50 μM competitor
+ 100 μM competitor
p18
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
CBP20
p18
1 2 3
E
m7GpppG
0 nM 1 nM 5 nM 10 nM 50 nM 100 nM
1 2 3 4 5 6
GpppG
0 nM 50 nM 100 nM 500 nM 1 μM 5 μM
7 8 9 10 11 12
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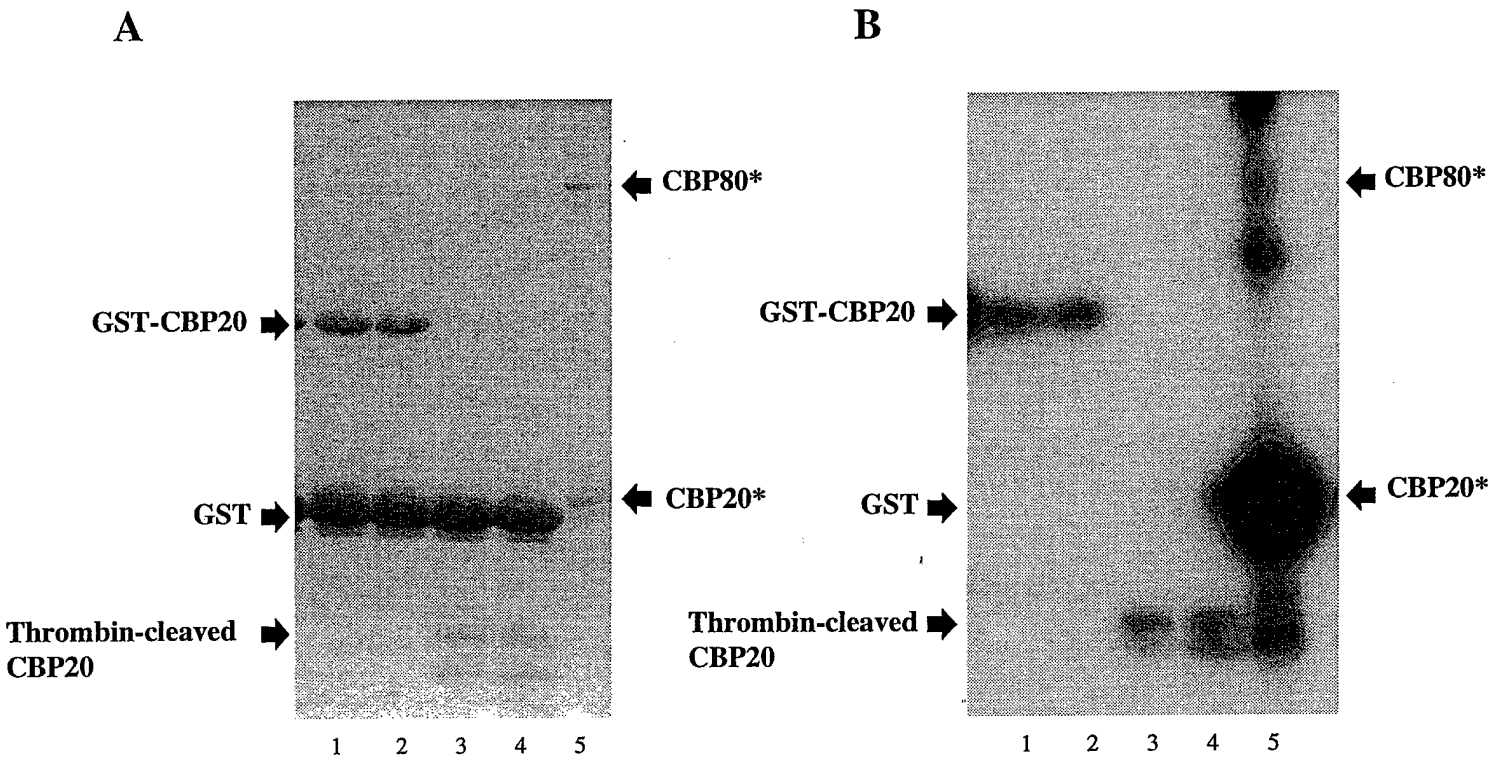
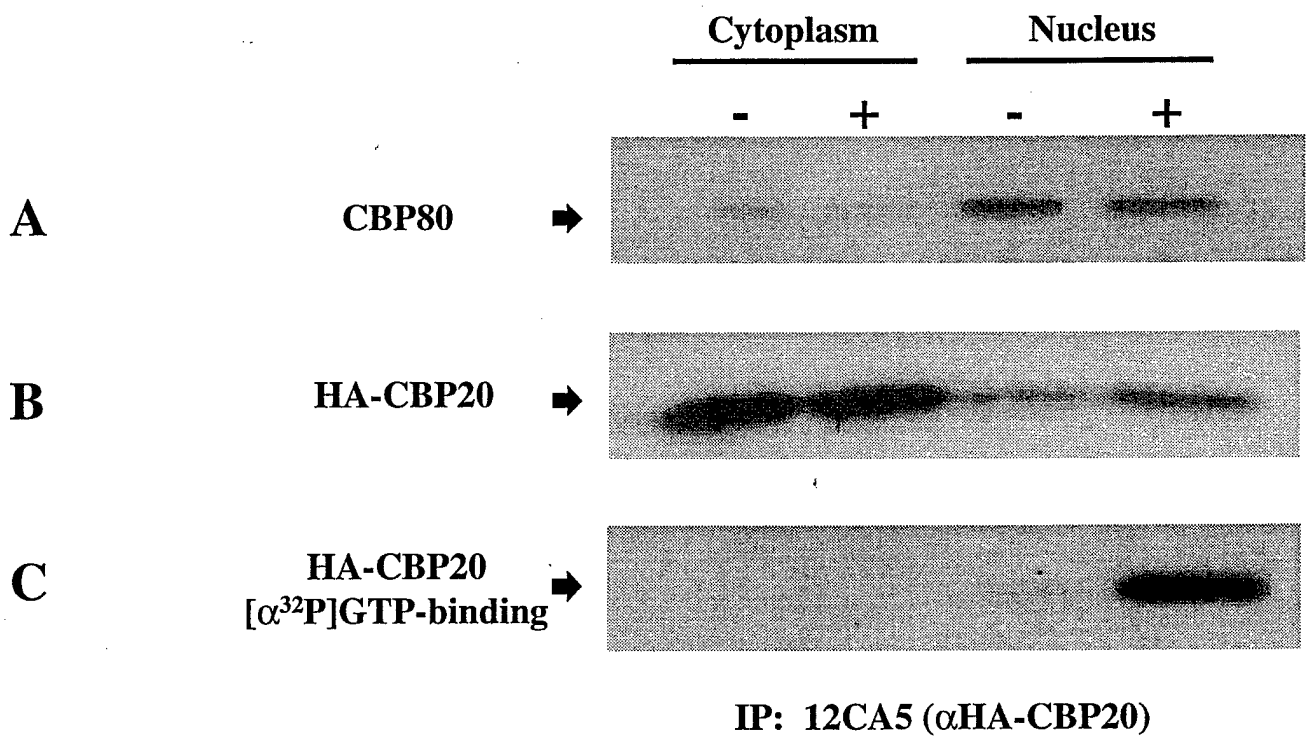
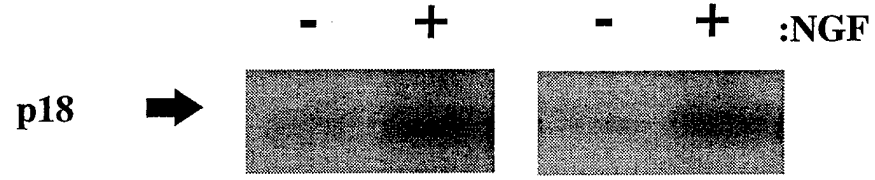
GTP
0 μM 0.5 μM 1 μM 5 μM 10 μM 50 μM
19 20 21 22 23 24

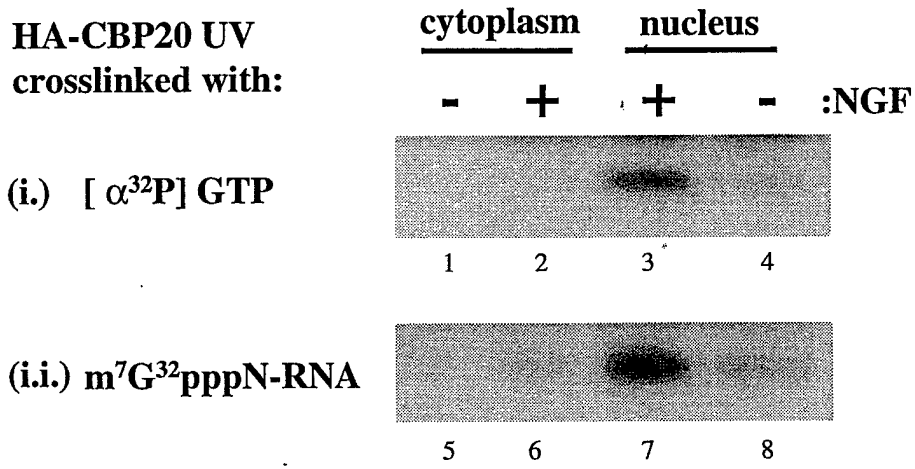

A
B
CBP80*
GST-CBP20
GST
CBP20*
Thrombin-cleaved
CBP20
1 2 3 4 5
CBP80*
GST-CBP20
GST
CBP20*
Thrombin-cleaved
CBP20
1 2 3 4 5


Cytoplasm
Nucleus
- + - +
A
CBP80
B
HA-CBP20
C
HA-CBP20
[α32P]GTP-binding
IP: 12CA5 (αHA-CBP20)

A


- + - + :NGF
p18
UV crosslinked with: [α32P] GTP m7G32pppN-RNA

B


HA-CBP20 UV crosslinked with:
cytoplasm
nucleus
- + + - :NGF
(i.) [α32P] GTP
1 2 3 4
(i.i.) m7G32pppN-RNA
5 6 7 8

A novel regulator of p21 activated kinases

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SUMMARY

Proteins of the p21 activated kinase (Pak) family have been implicated in the regulation of gene expression, cytoskeletal architecture and apoptosis. While the ability of Cdc42 and Rac GTPases to activate Pak is well established, relatively little else is known about Pak regulation or the identity of Pak cellular targets. Here we report the identification of two closely related Pak3 binding proteins, possibly arising from alternative splicing, designated p50 and p85Cool-1 (cloned out of library). Both isoforms of Cool contain an SH3 domain that directly mediates interaction with Pak3 and tandem Dbl homology (DH) and pleckstrin homology (PH) domains. Despite the presence of the DH-PH motif, a characteristic of Rho family activators, activation of Cdc42 or Rac by Cool is not detectable. Instead binding of p50Cool-1, but not p85Cool-1, to Pak3 represses its activation by upstream activators such as the Dbl oncoprotein, indicating a novel mechanism of regulation of Pak signaling.

INTRODUCTION

The Rho family GTPases Rac1 and Cdc42 mediate diverse biological events including changes in the cytoskeletal architecture (1-3), stimulation of DNA synthesis (4), cellular transformation (5-8) and signaling to the nucleus (9-14). Many of the signaling pathways leading to the execution of these events involve the p21-activated kinases, Paks1-3, which are direct effectors of Rac1 and Cdc42 (15-17). Binding of these GTPases to a conserved p21 binding domain (PBD, aka CRIB for Cdc42/Rac1 interactive binding) stimulates their serine/threonine kinase activities by a mechanism involving autophosphorylation (15, 18). The important roles that Paks play as effectors of Cdc42/Rac1 signaling have been established from genetic and biochemical studies in yeast and mammalian cells. The budding yeast homolog of mammalian Paks, Ste20, acts in concert with Cdc42 in the pheromone response to activate a MAP kinase cascade leading to transcription of genes required for cell cycle arrest. The same protein functions as an effector of Cdc42 to activate a different MAP kinase cascade leading to filamentous growth in response to nitrogen starvation (19). The yeast model in which Pak/Ste20 links Cdc42 to transcriptional and cytoskeletal events is paralleled in mammalian cells; constitutively activated Pak mutants can activate the Jnk MAP kinase cascade leading to transcriptional control (12, 13) and can mimic some, though not all, of the effects of Rac1 or Cdc42 on cytoskeletal organization (18, 20).

The apparent multiplicity of Pak-mediated signaling pathways suggests that Pak activity must be tightly regulated. This has been made all the more clear from the observations that Pak1 function is required for cellular transformation by Ras (10) and that Pak2 activation is involved in Fas-mediated apoptosis (21, 22). Here we describe the identification of two isoforms of a novel Pak binding protein, probably resulting from alternative splicing of the same message, one of which is able to suppress Pak activation by upstream regulators.

EXPERIMENTAL PROCEDURES

Yeast-two hybrid screen. To identify proteins that interact with Pak3, the yeast strain L40 was co-transformed with Pak3 fused to the LexA DNA binding domain, which regulates expression of both his3 and lacZ, and a HeLa cDNA library was fused with the Gal4 activation domain (23). Clones positive for β-galactosidase activity were rescued and several clones that provided bait-dependent his3 and lacZ gene activation were sequenced. A 1 Kb (Eco RI - Xho I) fragment of clone Y107 was used to screen an oligo dT-primed, size fractionated (4-9 Kb) U937 library (a gift from J. Burrows, Massachusetts Institute of Technology, Boston) and a HeLa cDNA library (Stratagene). The U937 library yielded a full length clone (A6) which was predicted to encode a protein of 436 amino acids; p50Cool-1. Several partial clones were recovered from the HeLa library that appeared to represent alternatively spliced forms of Y107. One of these (clone 12a) was identical to a recently cloned cDNA, p85SPR, but lacked the 3' end encoding its C-terminal 31 amino acids. The p85Cool-1 cDNA was generated by fusing this 3' end, derived from the 3' untranslated region of the U937 clone A6, to clone 12a (p85Cool-1 is therefore identical to p85SPR).

Plasmid construction. The coding sequence of Pak3 was excised as a 1700 bp BamH I fragment from plasmid pJ3HmPak3 (12) and subcloned into pLexA (23). A BamH I site (GGA frame) was engineered in Cool-1 in front of the initiation methionine and the BamH I-Xho I (1-730 bp) and Xho I-Bgl II (670 bp) fragments which include the stop codon were ligated into the BamH I site of pBS SK (Stratagene) to generate plasmid pBSA6Cool. The BamH I-EcoR I fragment from pBSA6Cool, encompassing the entire coding sequence of p50Cool-1, was subcloned into Myc-tagged eukaryotic expression plasmid CMV6M to express p50Cool-1. pCMV6M(p50Cool-1)W43K was constructed by three fragment ligation containing the (420 bp) BamH I-Xba I PCR product, the (971 bp) Xba I-EcoR I fragment from pBSA6Cool, and the (5000 bp) BamH I-EcoR I fragment from the CMV6M vector. Plasmid CMV6Mp85Cool (to express Myc-tagged p85Cool-1) was generated by ligating a BamH I-Bsg I (1610 bp) fragment

(from clone 12a) and a PCR generated (340 bp) Bsg I-EcoR I fragment (from the 3' untranslated region of U937 clone A6) into the BamH I-EcoR I site of the CMV6M vector. Plasmid pCMV6Dbl was generated by ligating a BamH I fragment encoding oncogenic Dbl from plasmid pc11dbl (a gift from Dr. Sandra Eva, Giannina Galini Institute, Geneva Italy) into the BamH I site of pCMV6. pCMV6HA-Cdc42 (HA-tagged Cdc42), pGEX-PBD (GST-PBD) and J3HmPak3 (HA-Pak3) have been previously described (12).

Kinase assays, affinity precipitation and immunoprecipitations. Kinase reactions were initiated by the addition of kinase buffer (40 mM Hepes, pH 7.4, 20 mM $MgCl_2$ and 4 mM $MnCl_2$) and 20 μM γ-[^{32}P]ATP (3000 Ci /mmol) for 3.5 min at room temperature. Reactions were stopped by the addition of 2X SDS sample buffer containing 20 mM EDTA. Affinity precipitation with GST-PBD was as described (24) except that COS cells were lysed in 25 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Lysates containing equal amounts of Pak3 were immunoprecipitated with anti-Pak3 primary antibody (a gift from Dr. S. Pelech, Kinetek Biotechnology Corporation, Vancouver)

Anti-Cool-1 antibody was prepared using His-tagged full length p50Cool-1, treated with thrombin to cleave the His-tag and further resolved on a Mono Q Sepharose column. Rabbit serum was collected 12 weeks after three injections with p50Cool-1.

RESULTS AND DISCUSSION

To identify potential Pak binding partners we used the yeast two-hybrid screen. One positive clone was used to screen cDNA libraries and databases (see Experimental Procedures) to yield two Pak3 binding proteins, p50 and p85Cool-1 (cloned out of library-1). They share an amino terminal SH3 domain (amino acids 7-65), followed by a Dbl homology (DH) domain (amino acids 100-279) and an adjacent pleckstrin homology (PH) domain (amino acids 295-400) (Figure 1A). Since p50 and p85Cool-1 are identical at the nucleotide level over amino acids 1-418, it is likely that they arise from alternative splicing of the same message. p85Cool-1 is identical to two recently cloned proteins; p85SPR (25) and β-PIX (26). The DH domain of the Cool proteins shows the highest sequence identity to the DH domains of Dbl (33%), Scd1 (30%), Dbs (29%), Tiam-1 (29%), Still Life2 (29%), Cdc24 (26%) and Vav2 (24%). In addition to the genetic screen, we used recombinant GST-Pak3 (amino acids 148-239) to purify Pak binding proteins from Src(Y527F)-transformed NIH 3T3 cells. Sequence obtained from an ~85 kDa Pak binding protein was identical to portions of p50 and p85Cool-1 (data not shown). We also identified a closely related cDNA from the databases and we have found that its product also interacts with Pak3[1]. This protein, Cool-2, is identical to the recently described α–PIX (26).

To determine whether the Cool proteins bind to Pak3 in mammalian cells, we transiently co-expressed Myc-tagged p50Cool-1 and hemaglutinin (HA)-tagged Pak3 in COS cells, and assayed for complex formation by immunoprecipitation and Western blot analysis (Figure 1B). HA-Pak3 was detected in anti-Myc immunoprecipitates (lane 8, upper panel) and Myc-p50Cool-1 was detected in anti-HA immunoprecipitates (lane 13, lower panel). Mutation of a conserved tryptophan residue within the SH3 domain (W43K) eliminated the ability of p50Cool-1 to associate with Pak3 (lane 10, upper panel and lane 14, lower panel), indicating that the SH3 domain of p50Cool-1 binds Pak3. The Src SH3 binding protein, Sam68, did not co-immunoprecipitate with Myc-p50Cool-1 (lane 9, upper panel), although it bound to the SH3 domain of Cool-1 *in vitro*[2] and SH3 domains from the Dbl family proteins Dbs and Vav did not bind Pak3 (data not shown),

showing specificity of the Cool-1/Pak3 association *in vivo*. Pak3 contains four conventional (PXXP motif) SH3 binding sites (P1-P4); Pak3 containing Pro to Ala mutations in the P1-P4 sites, alone or in combination, were used to establish that these sites do not mediate Pak3/Cool-1 interactions (data not shown). While this manuscript was in preparation, Manser et al. reported binding of α–PIX and β–PIX (which correspond to Cool-2 and p85Cool-1) to residues 182-203 of Pak1, confirming the atypical (non-PXXP motif) nature of Cool binding to Paks (26). Using a rabbit antiserum raised against full length p50Cool-1, we detected two predominant proteins migrating at ~85 kD and ~78 kD on SDS-PAGE in mouse NIH 3T3 fibroblast lysates (Fig. 1C, right panel, lane 1). Both of these proteins were detected in anti-Cool-1 immunoprecipitates (Fig. 1C, right, lane 3) as well as in anti-Pak3 immunoprecipitates (lane 2), demonstrating an interaction between endogenous Cool proteins and Pak3 in NIH 3T3 cells. The less reactive band at ~50 kD recognized by the anti-Cool-1 antibody (right panel, lane 1) may represent p50Cool-1; however, we were unable to determine whether p50Cool-1 was present in the immunoprecipitates because of the overlapping signal for IgG.

The presence of a DH-PH tandem motif in Cool-1, a hallmark of Dbl family exchange factors (27), initially led us to consider the possibility that Cool-1 might activate Cdc42 or Rac. However, we were unable to detect stimulation of [^{3}H]GDP dissociation from Cdc42 or Rac by recombinant p50Cool-1 purified from *E. coli* or insect cells under conditions where the Dbl oncoprotein strongly stimulated GDP dissociation from Cdc42 or RhoA (data not shown).

We then considered the possibility that Cool-1 exchange activity may require cellular co-factors or post-translational modifications as proposed for Vav, Sos and Tiam-1 (28-31). To measure Rac1 or Cdc42 activation *in vivo*, we used a modification of a recently described assay for activated, GTP-bound Ras (24). The p21(Cdc42/Rac)-binding domain (PBD) of Pak3 was expressed as a GST fusion protein and immobilized by binding to glutathione Sepharose beads. The immobilized GST-PBD was used to precipitate activated Cdc42 or Rac1 from transfected COS cell lysates (Figure 2). In untreated control cells, relatively low levels (< 5%) of HA-Rac1 (upper panel) or HA-Cdc42 (lower panel) were precipitated with GST-PBD [compare lane 1 (cell lysates)

and lane 9 (affinity-precipitated GTPase) in both panels]. When Dbl was co-expressed with HA-tagged GTPases, there was a marked increase in the amounts of activated HA-Rac1 and HA-Cdc42 that were precipitated with GST-PBD (lane 16). The cellular activation of both Cdc42 and Rac by Dbl is consistent with results from micro-injection studies (32). In contrast, p50Cool-1 did not detectably activate either HA-Rac1 or HA-Cdc42 (compare lanes 9 and 10), even after exposure to a number of different biological stimuli (lanes 11-15). Activation of Rac1 or Cdc42 by p85Cool-1 was also not detected, even when co-expressed with Pak3, under conditions where Tiam-1 resulted in the activation of Rac1 but not Cdc42 (data not shown). We have not detected Cool-1 mediated Rac1 activation in cells co-expressing potential activators of Rac including Src(Y527F), Ras(G12V) and Cdc42(Q61L)[2]. We next tested whether p50/p85Cool-1 could directly modulate Pak activity. While p85Cool-1 was without significant effect (Figure 3A, compare lanes 2 and 4), p50Cool-1 strongly inhibited Dbl-stimulated Pak3 activity (lanes 2, 3). p50Cool-1(W43K) did not inhibit Dbl-activated Pak3 (lanes 2, 5) indicating that binding of p50Cool-1 to Pak3 was required for its inhibitory effect. Immunoblotting confirmed that wild type and mutant Cool-1 proteins were equally expressed and that Dbl expression levels were unaffected by Cool-1 expression (data not shown); immunoprecipitated Pak3 levels were equal (Fig. 3A, lower panel). We also found that purified, recombinant p50Cool-1 completely blocked Cdc42(Q61L)-stimulated autophosphorylation of Pak3 and strongly inhibited the phosphorylation of MBP in Pak3 immunoprecipitates (data not shown).

We examined the effects of Cool-1 on binding of activated Cdc42 to Pak3 by expressing Myc-tagged Pak3 and/or p50Cool-1 in COS cells and assaying the binding of Pak3 to GST-Cdc42 by affinity precipitation and anti-Myc-immunoblotting (Figure 3B). Pak3 was precipitated by immobilized GST-Cdc42(Q61L) [GTPase-defective GTP-bound, (lane 6)] but not by GST-Cdc42 (GDP-bound) (lane 5) and displayed a gel mobility shift due to Cdc42(Q61L)-stimulated autophosphorylation (lanes 1, 6). [Pak3 autophosphorylation is sustained by the presence of Mg^{++} during affinity precipitation]. Although p50Cool-1 expression did not affect Pak3 recovery in GST-Cdc42(Q61L) precipitates (compare lanes 1, 6 with lanes 3, 10), the precipitated Pak3 did

not display the gel shift observed in the absence of p50Cool-1 or when co-expressed with p50Cool-1(W43K) (compare lanes 6, 10, and 12). Therefore, p50Cool-1 binding to Pak3 did not inhibit Cdc42 binding but did inhibit Cdc42-stimulated Pak3 autophosphorylation. Relative to Myc-Pak3, very little Myc-p50Cool-1 is detected in these precipitates (lane 10), which may be due to dissociation of p50Cool-1 from Pak3 during the precipitation and washing procedures, but may also reflect the relatively poor ability of anti-Myc to detect Myc-tagged p50Cool-1 co-precipitated with activated Cdc42 and Pak3 (see below).

Unlike p50Cool-1, expression of p85Cool-1 did not inhibit Cdc42(Q61L)-stimulated Pak3 autophosphorylation (Fig. 3C, compare lanes 8 and 9), which is consistent with the results shown in Fig. 3A. As expected from the presence of identical SH3 domains, p50 and p85Cool-1 competed for binding to Pak3 *in vitro* (lanes 9-12). In these experiments, p50Cool-1 co-precipitating with Pak3 was not detected with anti-Myc (upper panel, lanes 8, 10-12), but was readily detectable with an anti-Cool-1 antibody (lower panel, lanes 8, 10-12). The fact that an ~3 fold excess of either p50Cool-1 or p85Cool-1 was able to significantly inhibit the binding of the other to Pak3 suggests that they have similar affinities for Pak3.

Our results suggest that p50Cool-1, by competing with endogenous p85Cool-1 or other Cool proteins for binding to Pak3, might sequester Pak3 away from its site(s) of activation. On the other hand p85Cool-1, which has a permissive effect on the stimulation of Pak activation by Dbl or other Rho-family exchange factors, could play an important role in recruiting Pak to its sites of activation, possibly via its C-terminal region which is not present in p50Cool-1. In support of this model, our preliminary data indicate that p50Cool-1 has a diffuse cytoplasmic localization[3], while p85Cool-1 is concentrated at focal adhesions (25). Moreover, HeLa cell p85Cool-1 (β–PIX) has recently been shown to localize to focal complexes and appears to mediate Pak1 recruitment to these sites by activated Cdc42 (26). However, this would not account for the ability of p50Cool-1 to inhibit Cdc42-stimulated Pak3 activity *in vitro*. Since both p50Cool-1 and p85Cool-1 appear to bind to a common site on Pak3, the specific inhibition of Pak3 activity by p50Cool-1 may be due to the differences in the C-terminal regions of p50 and p85Cool-1.

Protein kinases are often subject to multiple levels of regulation. The involvement of Paks in cellular signaling pathways leading to changes in gene expression or cytoskeletal architecture and their participation in both Ras-mediated transformation (7-10) and Fas-mediated apoptosis (21, 22), mandates that their activities be tightly controlled. The ability of p50Cool-1 to suppress and p85Cool-1 to permit Pak activity indicates that signaling through Pak-dependent pathways may be regulated by cell type, cell cycle or developmental-specific expression patterns. It will be important to establish the role of DH and PH domains in Cool function and how differential expression of Cool impacts upon Pak signaling to different effector pathways.

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FOOTNOTES

[1]S. Bagrodia, M. Hart and R. Cerione unpublished observations.

[2]S.J. Taylor, S. Bagrodia, and R. Cerione unpublished observations.

[3]A. Ridley, S. Bagrodia and R. Cerione unpublished observations.

FIGURE LEGENDS

Fig. 1. Cool-1 is a Pak3 binding protein. *a.* Schematic representation of Cool-1. The coding region of p50Cool-1 containing the SH3, DH and PH domain is identical to that of p85Cool-1 which additionally contains a C-terminus with potential SH3-binding sites (PXXP) denoted by vertical lines. *b.* COS cells were co-transfected with the plasmids (1 μg of each) expressing the indicated HA- or Myc-tagged proteins. Anti-Myc (9E10) (lanes 6-10) and anti-HA (12CA5) (lanes 11-14) immunoprecipitates were Western blotted. The top (>~60 KDa) and bottom (<~60 KDa) parts of the blots were probed with anti-HA and anti-Myc antibodies respectively. Lanes 1-5 represent 10% of the whole cell lysates (WCL) used in the immunoprecipitation reactions. *c.* Endogenous proteins were immunoprecipitated from NIH 3T3 cell lysates (WCL, lanes 1) using anti-Pak3 (NT) polyclonal antibody (lanes 2, Pak3 IP), anti-Cool-1 polyclonal antibody (lanes 3, Cool-1 IP) or rabbit preimmune serum (lanes 4, pre). Proteins were Western blotted and blots were probed with anti-Pak3 or anti-Cool-1.

Fig. 2. **Cool-1 does not detectably activate Cdc42 or Rac1 *in vivo*.** COS cells were transfected with HA-Rac1 (top) or HA-Cdc42 (bottom) alone (lanes 1, 9) or co-transfected with Myc-p50Cool-1 (lanes 2-7 and lanes 10-15) or with Dbl (lanes 8 and 16) for 48 h and then serum-starved for another 4 h and treated with 100 ng/ml EGF plus 25 ng/ml IGF-1, 40 ng/ml PDGF, 20% fetal calf serum, 50 ng/ml IL-1 for 10 min or 200 μM methylmethane sulphonate (MMS) for 1 h as indicated. Cells were lysed and affinity-precipitated with immobilized GST-PBD (GST-PBD AP) (lanes 9-16) and bound proteins were Western blotted and probed with anti-HA. Lanes 1-8 represent 5% of the whole cell lysate (WCL) used in the binding reaction.

Fig. 3. **Cool-1 inhibits Dbl- and Cdc42-stimulated Pak3 activity.** *a.* COS cells were transiently co-transfected with Pak3 (0.5 μg), Myc-p50Cool-1 (1 μg), Myc-p85Cool-1 (1 μg), Myc-p50W43KCool-1 (1 μg), or Dbl (1 μg), and Pak3 kinase activity was measured in Pak3

immunoprecipitates using myelin basic protein (MBP) as a substrate. The top part of the gel was probed with anti-Pak3 and the bottom part of the blot was autoradiographed. Based on Western blot analysis the amount of Dbl was similar in lanes 2-5, the amounts of p50Cool-1 and p50Cool-1(W43K) were also similar in lanes 3 and 5 and p85Cool-1 was present in a slightly larger amount. *b*. COS cells were transiently transfected with 1 μg each of Myc-Pak3, Myc-p50Cool-1 or Myc-p50W43KCool-1. Cells were lysed and affinity-precipitated with immobilized GST-Cdc42 (lanes 5, 7, 9 and 11) or with constitutively active GST-Cdc42(Q61L) (lanes 6, 8, 10 and 12). Bound proteins were Western blotted and probed with anti-Myc. Lanes 1-4 represent 10% of the whole cell lysate used in the binding reaction. *c*. COS cells were transiently co-transfected with empty vector (lane 1) or plasmids encoding HA-Pak3 [lanes 2-6, (0.5 μg DNA)], Myc-p50 Cool-1 [lanes 1, 4, 6 (0.5 μg) and 5 (1.5 μg)], Myc-p85 Cool-1[lanes 3, 4 , 5 (0.5 μg) and 6 (1.5 μg)]. Cells were lysed and affinity-precipitated (AP) with immobilized GST-Cdc42L61 (~12 μg, lanes 7-12). Bound proteins were Western blotted and the blot was probed with anti-HA and anti-Myc. Lanes 7-12 of the blot were reprobed with anti-Cool-1 to detect p50 Cool-1. Lanes 1-6 represent 10% of the whole cell lysate (10% WCL) used in the binding reaction. Numbers above the lanes denote the ratio of p50: p85 Myc-tagged Cool-1 cDNA that was transfected.

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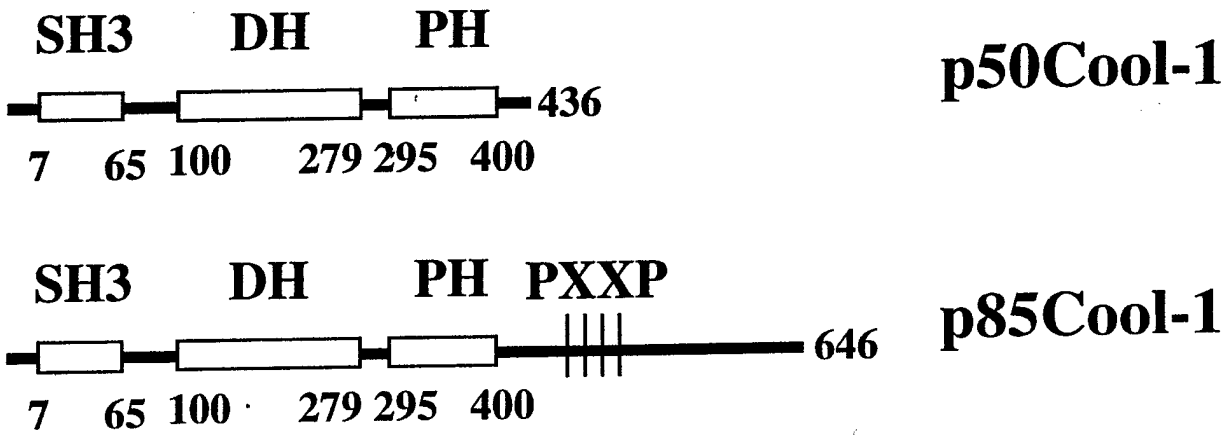
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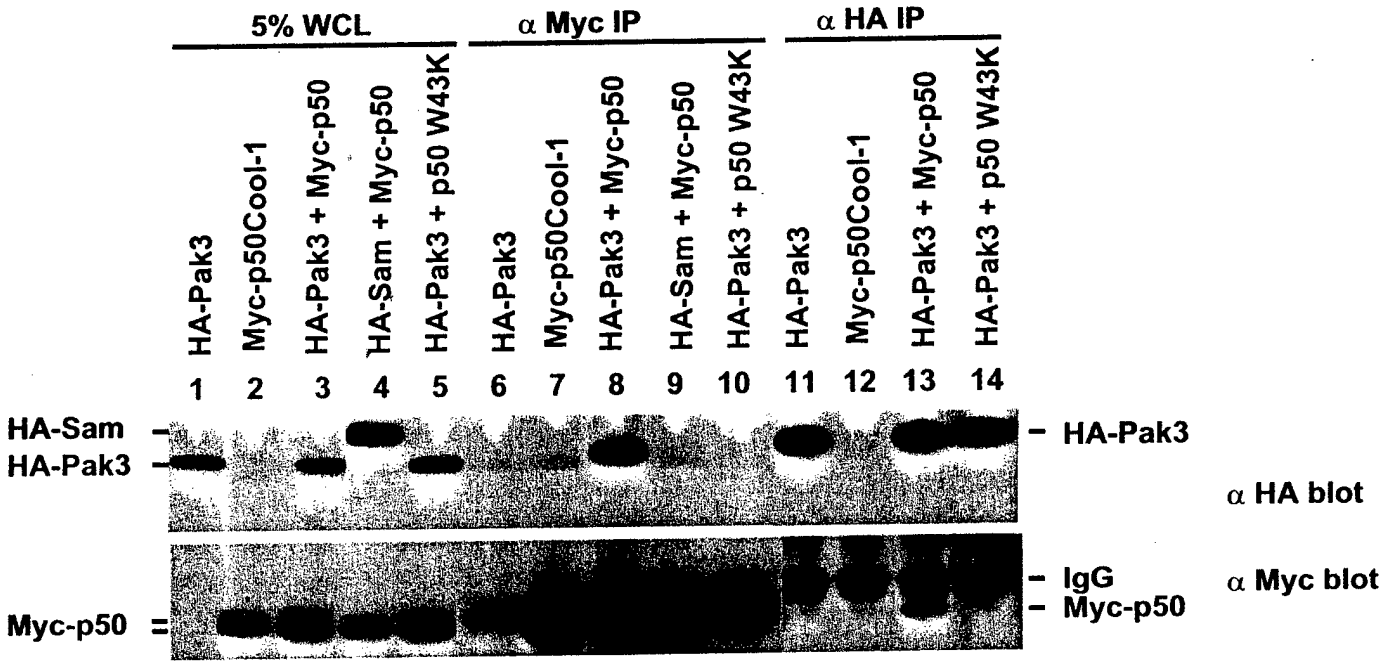
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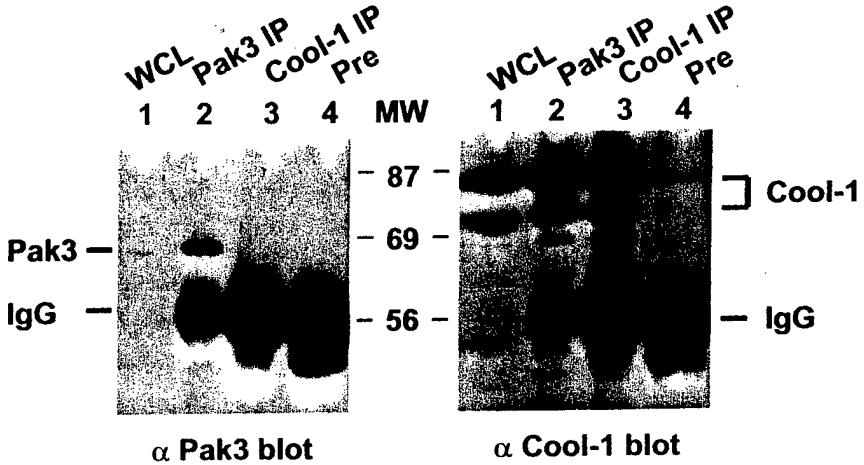
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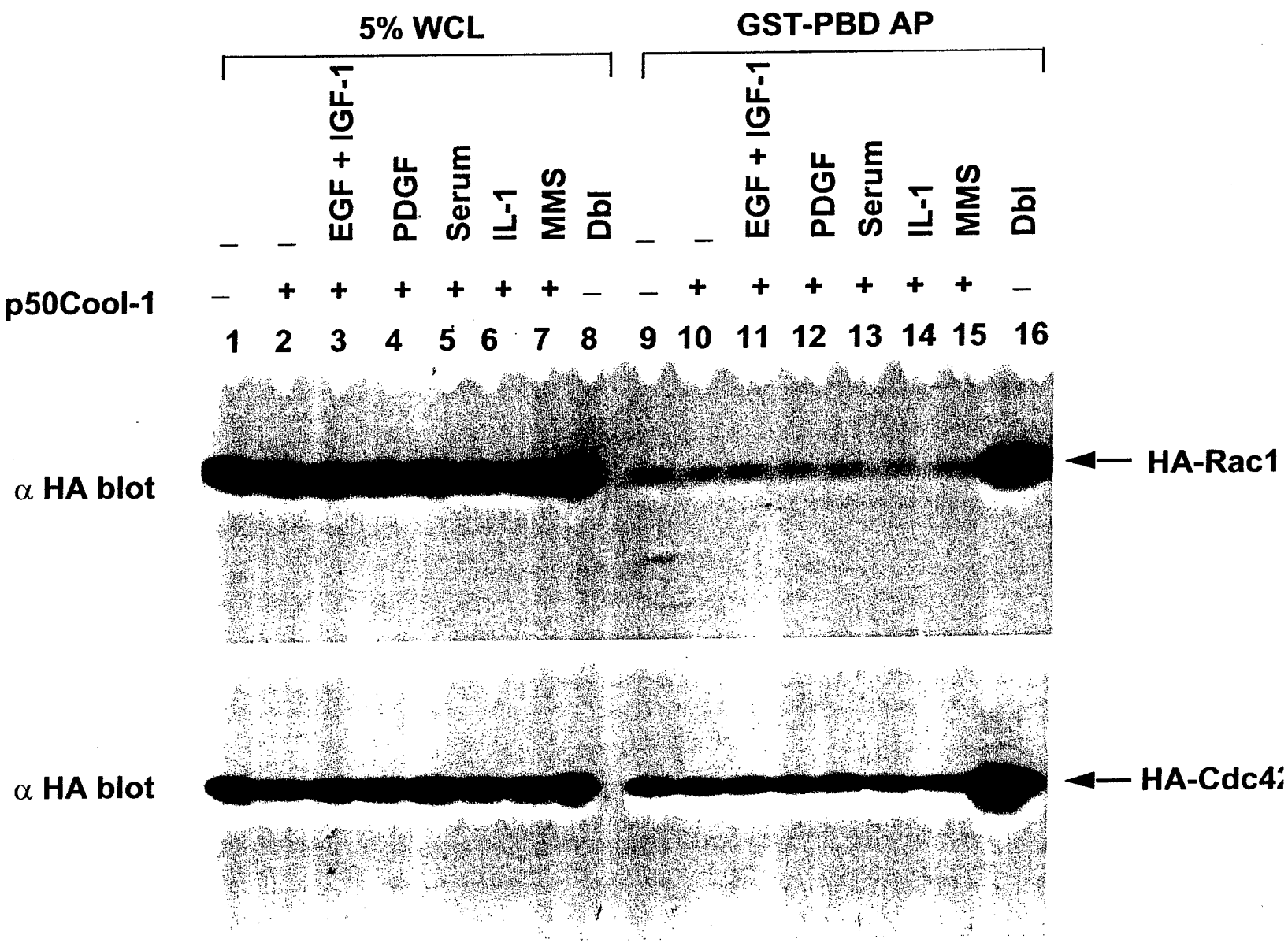
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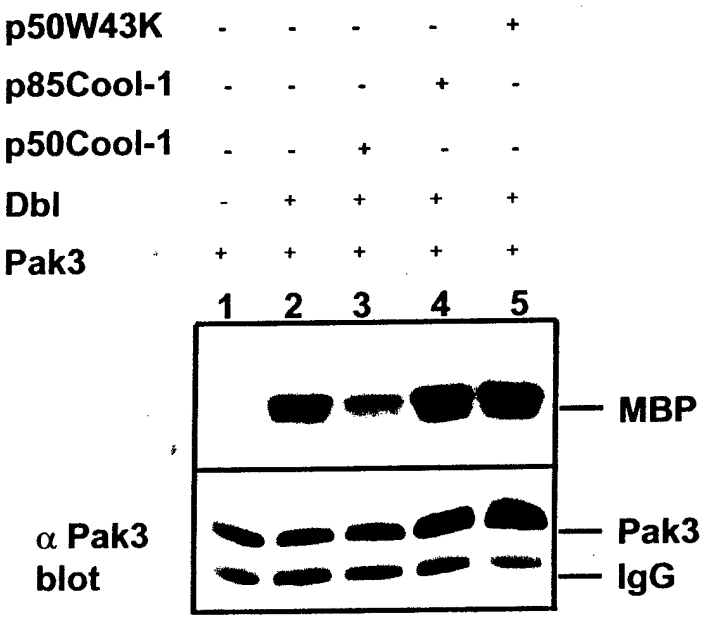
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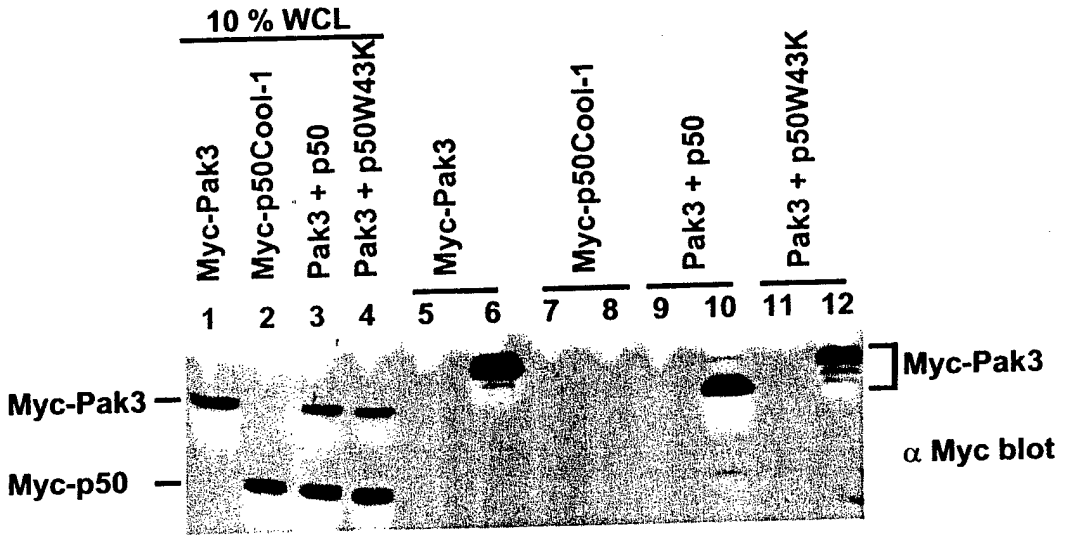
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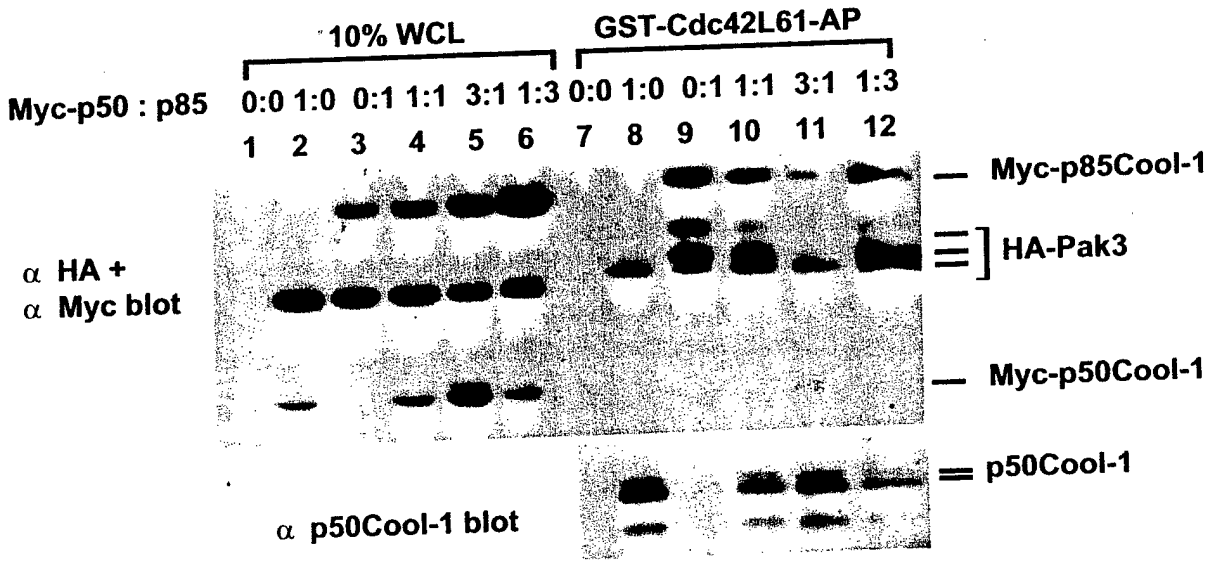
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